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Award Number: DAMD17-02-1-0155

TITLE: Selective Killing of Prostate Tumor Cells by Cytocidal Viruses

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REPORT DATE: February 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20050727 126

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE February 2005	3. REPORT TYPE AND DATES COVERED Final (1 Feb 2002 - 31 Jan 2005)

4. TITLE AND SUBTITLE
Selective Killing of Prostate Tumor Cells by Cytocidal Viruses

5. FUNDING NUMBERS
DAMD17-02-1-0155

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The goal was to develop novel vectors for therapy of prostate tumors based on vesicular stomatitis virus (VSV). The novelty in our approach is our ability to enhance the selectivity of VSV-induced killing of tumor cells versus normal cells by manipulating the viral genes that control the antiviral interferon response. Aim 1 was to identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells. Aim 2 was to identify VSV mutants that enhance the antiviral interferon response in prostate cells. Aim 3 was to determine whether VSV mutants have greater efficacy and safety than wild-type VSV in reducing prostate tumors in nude mice. We identified VSV mutants with enhanced ability to kill prostate tumor cells versus normal prostatic epithelial cells in culture (Aim 1). We showed that the same mutant had enhanced ability to induce antiviral interferon responses (Aim 2), and was an effective killer of interferon-nonresponsive prostate tumors established in nude mice. However, we found that other prostate tumors are responsive to interferons, and are resistant to treatment with oncolytic VSV in vivo. These data suggest that screening of prostate tumors for susceptibility may be necessary prior to treatment with oncolytic VSV.

14. SUBJECT TERMS
Virual vector, gene therapy, apoptosis, interferons

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

The goal of this project is to develop novel vectors for therapy of prostate tumors based on vesicular stomatitis virus (VSV). VSV kills many tumor cells more effectively than normal cells, due in part to defects in the antiviral response in tumor cells. The novelty in our approach is our ability to enhance the selectivity of killing of tumor cells versus normal cells by manipulating the viral genes that control the antiviral interferon response. Aim 1 was to identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells. Aim 2 was to identify VSV mutants that enhance the antiviral interferon response in prostate cells. Aim 3 was to determine whether VSV mutants have greater efficacy and safety than wild-type VSV in reducing prostate tumors in nude mice. In the previous reporting period, we had largely completed the experiments in Aims 1 and 2 for normal prostate cells and the tumor cells (LNCaP and PC-3), and identified several mutant viruses with the desired properties. In the final reporting period, we have completed Aim 3, indicating that one of the mutant viruses is an effective, and safer vector for treating LNCaP tumors in nude mice. In addition, during the present reporting period, we have analyzed tumor cells cultured from patient prostatectomy specimens as well as the more resistant PC3 tumor cell line. These results have shown that there is considerable variability in the response of prostate tumor cells to oncolytic VSV, such that some tumors are likely to be susceptible while others are likely to be resistant. The results of this study have been published in a paper in *Virology* (Ahmed, Cramer, and Lyles, 2004), which is included in the Appendix.

Body:

Aim 1: To identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells.

Accomplishments for Aim 1:

We tested five different viruses for their ability to infect and kill tumor and benign prostate cells. These experiments were done to show which mutations enhance differential killing of tumor versus normal cells by VSV. Wild-type (wt) VSV strains included the naturally occurring Orsay strain (wtO), which has been studied in research laboratories for many years, and a recombinant wild-type (rwt) virus derived from an infectious cDNA clone modified slightly (Kopecky, Willingham, and Lyles, 2001) from the one described by Whelan et al. (Whelan et al., 1995). This virus has genes derived from several different strains of VSV. The mutant viruses contained point mutations in the M protein. The matrix (M) protein of VSV serves two major classes of functions in virus-infected cells (reviewed in (Lyles, 2000)). One class of functions are involved in assembly of progeny virions. The other class of functions is involved in suppressing host gene expression, particularly the induction of antiviral responses. We have shown previously that a point mutation substituting methionine for arginine at position 51 of the 229 amino acid M protein (M51R mutation) renders M protein defective in its ability to suppress host antiviral responses, but the mutant M protein is as effective as wt M protein in the virus assembly functions necessary for virus replication (Ahmed and Lyles, 1997; Ahmed et al., 2003; Black et al., 1993; Kaptur et al., 1995). RM51R-M virus is a recombinant virus that is isogenic with rwt virus except for the M51R mutation. The r1026-M virus and tsO82 virus are similar M51R-M protein mutants on different strain backgrounds (Ahmed et al., 2003). R1026 virus is a recombinant virus that is isogenic with rM51R-M virus, except the M protein is derived from the HR strain, and tsO82 virus is a naturally occurring M51R M protein mutant derived from wtO virus. These viruses have somewhat different growth properties and abilities to induce cell death by apoptosis, so we tested the two wt and three M protein mutants for their ability to infect and kill tumor and benign prostate cells. As a result of the experiments in task 1, we chose to focus on two viruses: the rwt and rM51R-M viruses. In some experiments we included wtO as a representative naturally occurring virus, which replicates to higher titers than the recombinant viruses.

Our hypothesis was that wt viruses should infect and kill both tumor and benign cells, whereas M protein mutants should only infect and kill tumor cells. In principle, the antiviral responses induced by the M protein

mutant viruses should protect benign cells, but the tumor cells should be susceptible to these viruses because they are defective in their antiviral responses. Our initial experiments in task 1 used time-lapse microscopy to assay the induction of apoptosis by morphological changes in infected cells. These experiments were suggestive, but did not fully support our hypothesis. Therefore, we used other assays for cell death to test this hypothesis. In addition, in response to the review of our manuscript, we performed experiments to test the effect of single-cycle versus multiple-cycle infection on virus yield and cell death. We also extended these experiments to a second strain of benign prostatic cells. These results served to strengthen our conclusions.

LNCaP and benign prostate cells are susceptible to infection with wt and mutant viruses under single cycle infection conditions. To determine the effectiveness of M protein mutant viruses as oncolytic viruses for prostate tumor therapy, we tested the ability rM51R-M virus to infect LNCaP tumor cells as well as benign human prostatic epithelial cells in single cycle infection experiments. LNCaP cells and primary cultures of benign prostate cells obtained from patients were infected with wt and mutant viruses and were analyzed for VSV antigen expression, production of infectious viral progeny, and the ability of viruses kill infected cells (Fig. 1 and 2).

The efficiency of infection with wt and mutant viruses was measured by determining the percentage of cells expressing the viral G protein surface antigen by flow cytometry. LNCaP cells and benign prostatic cells (WFU55PZ strain) were infected with wt and rM51R-M viruses at a multiplicity of 10pfu/cell, so that in theory nearly 100% of cells were infected. At 4 and 8 hr postinfection, cells were fixed and incubated with antibody to G protein and a fluorescein-conjugated secondary antibody, then analyzed by flow cytometry. Results in Fig.
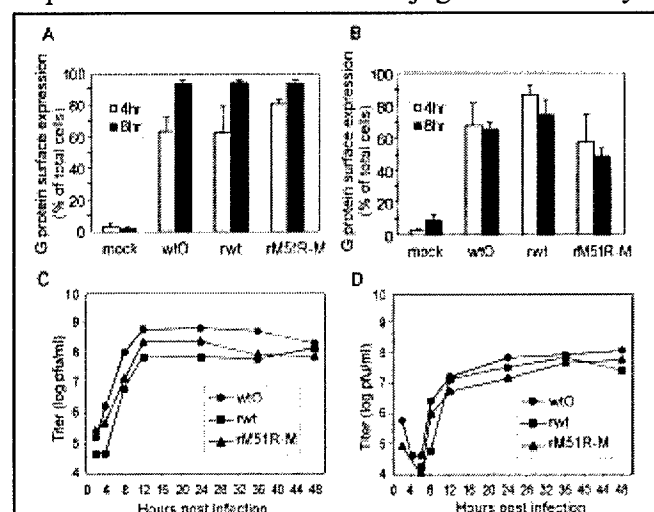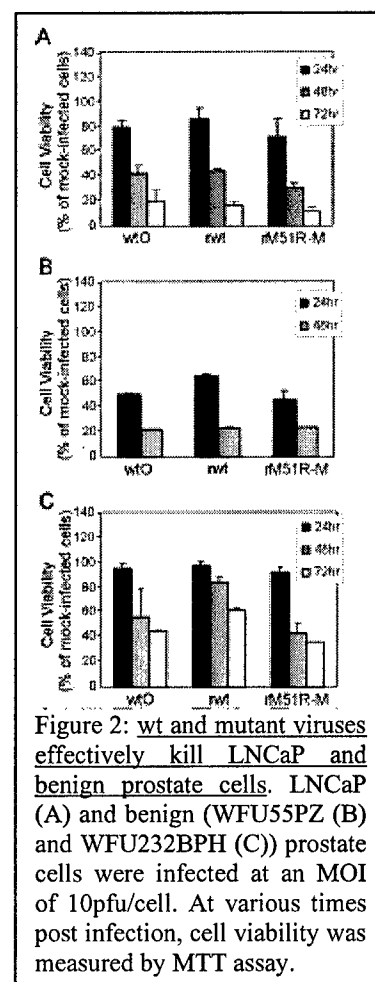

Figure 1: LNCaP and benign prostate cells are sensitive to VSV infection under single cycle conditions. A, B. Viral G protein expression in cells infected with wt or mutant viruses was measured by flow cytometry. C, D. Infectious progeny virus in infected cells was determined by plaque assay.

1A show that between 90 and 100% of LNCaP cells infected with wtO, rwt, and rM51R-M viruses expressed G protein at the cell surface by 8 h post-infection. These data indicate that there is no difference between rM51R-M virus and the wt viruses in their efficiency of infection of LNCaP tumor cells. Similarly, 70-90% of WFU55PZ cells infected with wtO or rwt viruses expressed the viral G protein. WFU55PZ cells infected with rM51R-M expressed G protein to a slightly lesser extent (50-60% of cells) than cells infected with wtO and rwt viruses (Fig. 1B) although this difference was not statistically significant. These data indicate that LNCaP cells and benign prostatic cells are similar in their susceptibility to VSV infection, and that there is little if any difference between viruses with wt and mutant M proteins in their ability to infect these cells.

The ability of wt and M protein mutant viruses to produce infectious progeny in LNCaP and WFU55PZ cells was determined by plaque assay (Fig. 1C and D). In LNCaP cells, wtO virus grew to titers approaching 10^9 pfu/ml, similar to results with other susceptible cell lines. Rwt virus grew to approximately one log lower titers, which is also similar to results in other cell types (Ahmed et al., 2003; Kopecky, Willingham, and Lyles, 2001). Variability between titers in repeated experiments was less than half a log. Thus, this difference between naturally occurring and recombinant viruses was statistically significant at most time points. RM51R-M virus grew to titers intermediate between those of rwt and wtO viruses, indicating that there is no defect in virus growth resulting from the M protein mutation. All three viruses produced similar levels of infectious progeny in WFU55PZ cells (Fig. 1D), although the titers of rM51R-M virus were slightly less than those of rwt virus at most times post-infection. Despite the difference between LNCaP and WFU55PZ cells in virus titers, these data indicate that both types of cells are permissive for virus replication.

The ability of wt and M protein mutant viruses to kill prostate tumor and benign cells was determined by MTT assay, which determines the metabolic activity of viable cells. These experiments compared LNCaP and WFU55PZ cells, and another benign prostate cell strain isolated from a patient with benign prostatic hyperplasia, WFU232BPH. Cells were infected at an MOI of 10 pfu/cell with each of the viruses and cell viability was measured at different times postinfection (Fig. 2). Results show that LNCaP cells were sensitive to killing by wt and mutant viruses and that there was no difference between wt and mutant viruses in their ability to kill these cells (Fig. 2). Similarly, WFU55PZ benign prostatic epithelial cells were sensitive to killing by each of the viruses so that by 48 h postinfection, approximately 20% of infected cells remained viable (Fig. 2B). WFU232BPH cells were somewhat more resistant to virus-induced cell killing, with approximately 40% of cells remaining viable at 72 h postinfection (Fig. 2C). Despite this difference between prostate cells from different patients, we can conclude from figures 1 and 2 that both prostate tumor and benign cells are susceptible to infection with wt and mutant viruses under single cycle infection conditions, and that M protein does not play a significant role in VSV-induced cell killing in the prostate. Further studies indicated that there was no difference between LNCaP and benign prostate cells in their susceptibility to VSV-induced apoptosis as measured by TUNEL assay and that rM51R-M virus was as effective as wt viruses at inducing apoptosis these cells in single cycle infections (data not shown).

Benign prostate cells are more resistant to infection with M51R-M virus than wt viruses in multiple cycle infection experiments. To determine whether antiviral responses affect spread of virus to surrounding cells, we tested the ability of wt and mutant viruses to infect LNCaP and benign tumor cells under multiple cycle infection conditions. We used a multiplicity of infection of 0.1pfu/cell, so that approximately 10% of cells were initially infected, and growth of virus depends on spread to surrounding uninfected cells. Similar to data obtained under single cycle infection conditions, rM51R-M grew to titers intermediate between those of rwt and wtO viruses in LNCaP cells in the multiple cycle growth experiment (Fig. 3A). Although all viruses reached titers as high as those observed in the single cycle growth experiment (Fig. 1C), there was a delay in growth due to differences in kinetics of virus spread. This delay was also seen in WFU55PZ cells infected with each of the viruses (Fig. 3B). However, in contrast to LNCaP cells, rM51R-M virus produced lower levels of infectious virus progeny than wt viruses in WFU55PZ cells. In fact, by 96 h post-infection, rM51R-M virus titers were greater than two logs lower than those of rwt and wtO viruses. Similar data were obtained from WFU232BPH benign prostate cells (data not shown). These data suggest that the elevated antiviral response in benign prostate cells infected with rM51R-M virus limits virus spread to surrounding cells.

Figure 2: wt and mutant viruses effectively kill LNCaP and benign prostate cells. LNCaP (A) and benign (WFU55PZ (B) and WFU232BPH (C)) prostate cells were infected at an MOI of 10pfu/cell. At various times post infection, cell viability was measured by MTT assay.

The ability of wt and mutant viruses to kill LNCaP and benign prostate cells under multiple cycle infection conditions was measured by MTT assay. Data indicate that LNCaP cells are equally susceptible to killing by each of the viruses (Fig. 3C). However, both isolates of benign prostate cells were highly resistant to cell killing by rM51R-M virus. Close to 90% of benign prostate cells infected with rM51R-M virus retained viability at 96 h post-infection (Fig. 3D and E). In contrast, both cell strains were sensitive to killing by rwt virus. WFU232BPH, but not WFU55PZ cells, were also sensitive to killing by wtO virus. In contrast to data obtained in the single cycle experiments, primary cultures of benign prostate cells are consistently resistant to infection with rM51R-M virus in multiple cycle infection experiments, likely due to the ability of this virus to induce an effective antiviral response in these cells as compared to LNCaP cells. The observation that WFU55PZ cells were also resistant to wtO virus infection at low multiplicity of infections suggests that these cells are also able to mount an antiviral response
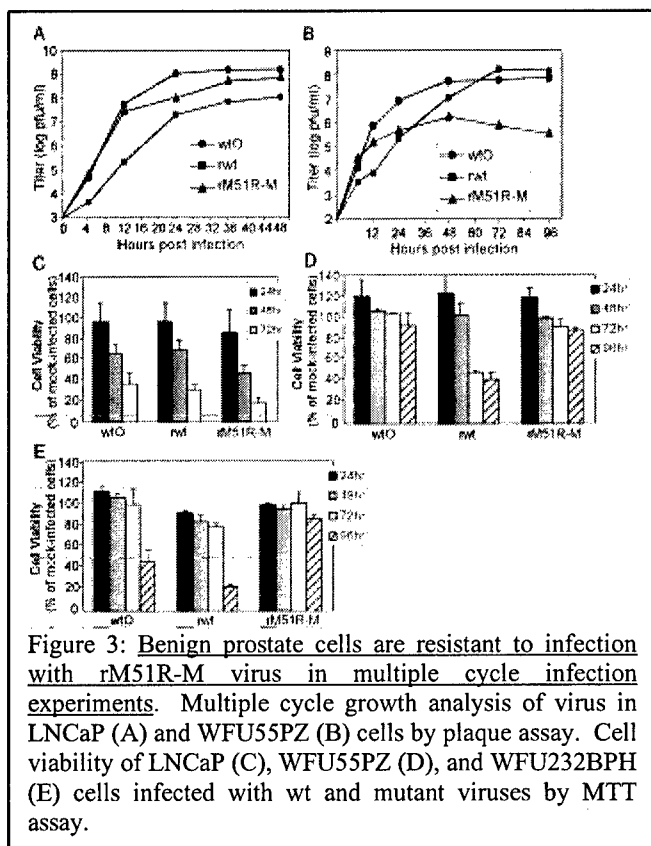
against wtO virus.

Figure 3: Benign prostate cells are resistant to infection with rM51R-M virus in multiple cycle infection experiments. Multiple cycle growth analysis of virus in LNCaP (A) and WFU55PZ (B) cells by plaque assay. Cell viability of LNCaP (C), WFU55PZ (D), and WFU232BPH (E) cells infected with wt and mutant viruses by MTT assay.

To summarize the results of these cell culture experiments, these data show that neither benign nor tumor prostate cells are inherently resistant to VSV infection and killing in single-cycle experiments. Furthermore, the M protein mutation had little if any effect on the ability of VSV to induce apoptosis in prostate cells. In contrast to single-cycle infection, multiple-cycle replication of the M protein mutant virus was severely restricted in benign prostate cells, but not in tumor cells. Thus the M protein mutant virus showed a greater selectivity for tumor cells versus normal cells than its wt control under conditions of multiple-cycle virus growth.

Human prostate cancer cell lines differ in their sensitivity to VSV infection. The main idea governing the selectivity of anti-tumor therapies using VSV is that many tumor cell lines are unresponsive to the anti-proliferative effects of IFN and should be correspondingly susceptible to VSV infection. However, we have found that human prostate cancer cell lines vary in their susceptibilities to VSV infection as well as their responsiveness to IFN. We compared the efficiency of infection of LNCaP cells as well as another prostate tumor cell line, PC-3, with wt and mutant viruses. The efficiency of infection was measured by determining the percentage of cells expressing the viral G protein surface antigen by flow cytometry. Between 90 and 100% of LNCaP cells infected with wtO, rwt, and rM51R-M viruses expressed G protein at the cell surface by 8 h post-infection. However, in contrast, PC-3 cells were highly resistant to infection with VSV, with only 10-30% of cells expressing the viral G protein. To determine whether the degree of infectivity correlated with VSV-induced apoptosis, LNCaP and PC-3 cells were infected with wtO, rwt and rM51R-M viruses and cells were analyzed for caspase-3 enzymatic activity. Results clearly showed that VSV-infected LNCaP cells were apoptotic as indicated by high caspase-3 activity while PC-3 cells had very low levels of caspase-3 activity. Taken together, the data indicate that PC-3 cells are resistant to infection with VSV while LNCaP cells are highly sensitive to VSV-induced cell killing, and that there is little if any difference between viruses with wt and mutant M proteins in their ability to infect these cells.

We analyzed the steps in the virus replication cycle that may be delayed or altered in VSV-resistant cells. Figure 4 shows that the rates of viral protein synthesis are lower in PC-3 cells as compared to LNCaP cells. For this experiment, cells were infected with wt and mutant viruses at a multiplicity of 10pfu/cell. At various times post infection, cells were incubated with ^{35}S-methionine for 15 min, harvested and lysed. Lysates were subjected to SDS-PAGE and the labeled viral nucleocapsid (N) protein was quantitated by phosporimaging. N protein expression is expressed as a percentage of the wtO N protein at 4 h for LNCaP cells and at 12 h for PC-3 cells, at which time
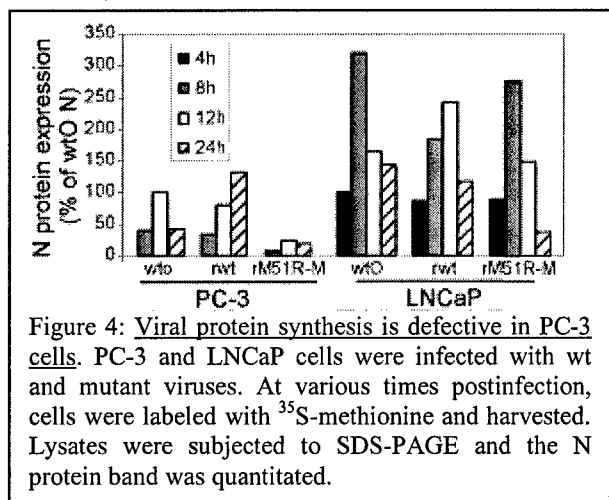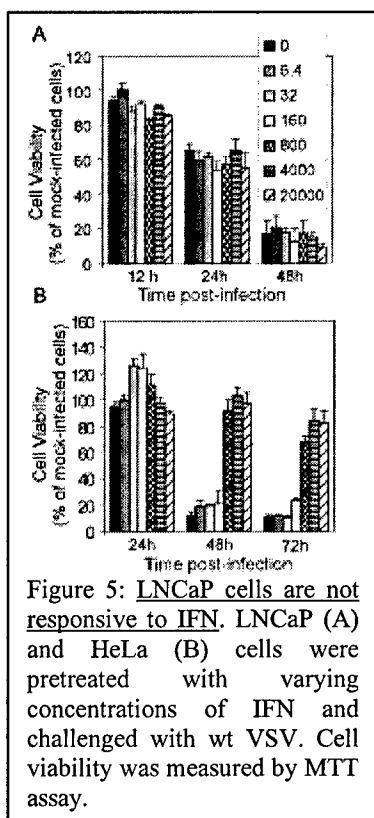

Figure 4: Viral protein synthesis is defective in PC-3 cells. PC-3 and LNCaP cells were infected with wt and mutant viruses. At various times postinfection, cells were labeled with ^{35}S-methionine and harvested. Lysates were subjected to SDS-PAGE and the N protein band was quantitated.

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equivalent levels of protein were observed for LNCaP and PC-3 cells. Results indicate that viral protein synthesis in VSV-sensitive LNCaP cells reaches a peak at 8 h, after which rates decrease due to VSV-induced cytopathic effect. The rates of protein synthesis in LNCaP cells are similar to those observed in cells displaying signs of robust VSV infection, such as HeLa and BHK cells. However, the rates of N protein synthesis in PC-3 cells are much lower than those in LNCaP cells. These results suggest that the antiviral response serves to inhibit viral protein synthesis in PC-3 cells.

We have extended these experiments to include secondary cultures of prostate tumor cells and determined the ability of each of the viruses to infect and kill these cells. These cells were intermediate in their sensitivity between LNCaP and PC3 cells. Collectively these results indicate that individual prostate tumors are likely to vary in their response to VSV infection.

Main conclusions from Aim 1:

1. Neither benign nor tumor prostate cells are inherently resistant to VSV infection and killing in single-cycle experiments.
2. The M protein mutation had little if any effect on the ability of VSV to induce apoptosis in prostate cells.
3. In contrast to single-cycle infection, multiple-cycle replication of the M protein mutant virus was severely restricted in benign prostate cells, but not in LNCaP tumor cells. Thus the M protein mutant virus showed a greater selectivity for tumor cells versus normal cells than its wt control under conditions of multiple-cycle virus growth.
4. LNCaP and PC-3 prostate tumor cells are differentially susceptible to infection by wt and mutant viruses. LNCaP cells are extremely sensitive to virus infection and killing, whereas PC-3 cells are relatively resistant.

Figure 5: LNCaP cells are not responsive to IFN. LNCaP (A) and HeLa (B) cells were pretreated with varying concentrations of IFN and challenged with wt VSV. Cell viability was measured by MTT assay.
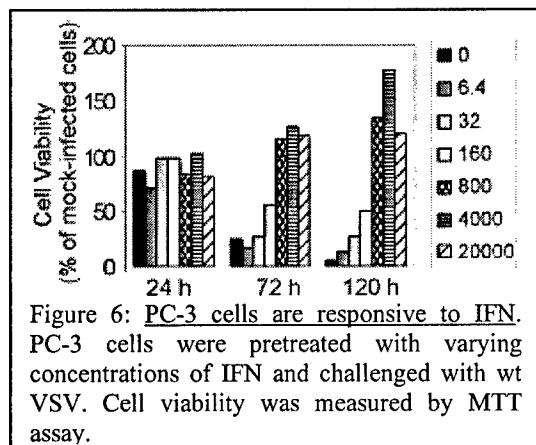
Aim 2: To identify mutations in VSV genes that enhance the antiviral interferon response in normal prostatic epithelial cells versus prostate tumor cells.

Accomplishments for Aim 2:

A main goal of this project is to develop viruses that have a high degree of selectivity for infecting tumor cells and not normal cells. At the point where these viruses may be used in cancer patients, this greater selectivity will result in viruses that are safer for use in patients, by virtue of their inability to replicate in normal tissue, but these viruses should still retain their effectiveness for replication and killing in tumor tissue. Our hypothesis is that M protein mutant viruses that induce interferon would be safer vectors due to their ability to induce an effective antiviral response in normal cells. To test this *in vitro*, we determined the level of interferon production by tumor cells versus normal cells by an interferon bioassay. The results showed that interferon activity was detected in each of these cell lines infected with the M protein mutant viruses (tsO82, rM51R and r1026) to varying degrees. Our data indicate that rM51R virus was the most effective inducer of interferon.

LNCaP prostate tumor cells are unresponsive to IFN. Clinical studies indicate that metastatic prostate tumors are not responsive to IFN treatments. (DiPaola and Aisner, 1999; Tjoa and Murphy, 2000) Figure 5 illustrates this lack of responsiveness using the LNCaP cell line. LNCaP cells were incubated with

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varying concentrations of IFN for 16h and challenged with VSV. At different times postinfection, cell viability was measured by MTT assay. Cells responsive to IFN should exhibit an IFN dose-dependent resistance to virus infection, while cells that are not responsive to IFN should show no difference in cytopathic effect regardless of IFN concentration. HeLa cells were used as a control since they are known to respond to IFN. Data in figure 5A show a decrease in LNCaP cell viability over the time course of the experiment due to virus infection. Furthermore, the results indicate that LNCaP cells are not responsive to IFN since they were not protected from VSV infection by pretreatment with IFN concentrations as high as 20,000 IU/ml. In contrast, HeLa cells were responsive to IFN (Fig. 5B) since close to 100% of cells displayed resistance to VSV infection upon pretreatment with concentrations of IFN $\geq$ 800 IU/ml.

Figure 6: <u>PC-3 cells are responsive to IFN</u>. PC-3 cells were pretreated with varying concentrations of IFN and challenged with wt VSV. Cell viability was measured by MTT assay.

In a separate series of experiments, we tested the responsiveness of PC-3 cells to IFN, PC-3 cells were incubated with varying concentrations of IFN for 16h and challenged with VSV at an MOI of 50pfu/cell. At different times postinfection, cell viability was measured by MTT assay. First of all, Figure 6 shows that PC-3 cells were resistant to VSV infection since all cells remained viable by 24 h postinfection. However, they were also responsive to IFN since close to 100% of cells displayed resistance to VSV infection upon pretreatment with concentrations of IFN $\geq$ 800 IU/ml by 72 h postinfection.
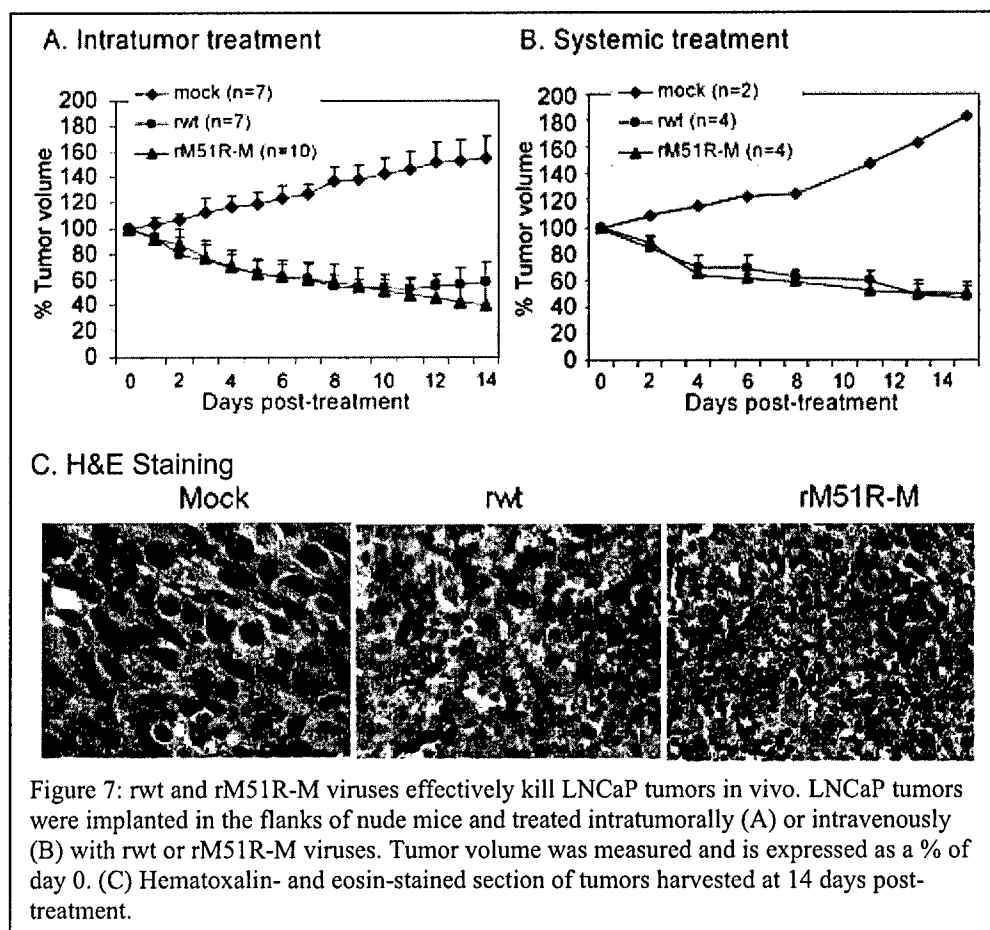
Main conclusions from Aim 2:

1. M protein mutant viruses induce interferon in normal prostate, PC-3 and LNCaP cells, whereas wt viruses do not.
2. LNCaP and PC-3 cells are differentially responsive to interferon. LNCaP cells, similar to many tumor cells, are not responsive to interferon. PC-3 cells are responsive to interferon.
3. The M protein mutant virus, rM51R, induces the highest levels of interferon in normal prostate cells.

Aim 3: To determine whether VSV mutants have greater efficacy and/or safety than wt VSV in reducing prostate tumors in nude mice.
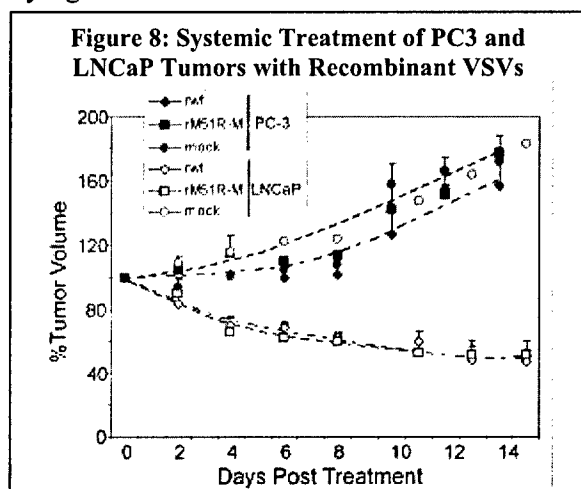
Accomplishments for Aim 3:

<u>Rwt and rM51R-M viruses effectively kill tumor cells *in vivo*.</u> To determine the ability of wt and mutant viruses to kill tumor cells *in vivo*, LNCaP prostate tumor cells were injected subcutaneously in the flanks of BALB/c nude mice. When palpable tumors were obtained, rwt and rM51R-M viruses (10^8 pfu) were injected intratumorally, and tumor volume was measured daily. Results in figure 7A show that tumor volume decreased in animals treated with both rwt and rM51R viruses, while mock-treated tumors continued to increase in size. By two weeks post-treatment, the tumors of mice injected with rM51R-M virus had decreased to approximately 30% of their initial volume at the start of the experiment. Interestingly, although the tumors of mice treated with rwt virus also regressed, some tumors started to increase in size again by ten days post-treatment. Although there was little if any difference between rM51R-M and rwt viruses in their effects on tumors, there was a striking difference in the morbidity of treated mice. 71% of mice treated with the rwt virus showed severe symptoms of VSV infection, including paralysis, weight loss and disorientation and were sacrificed between days 7 and 14. However, none of the mice treated with rM51R-M virus showed signs of illness and there was complete elimination of tumors by 6 to 8 weeks in some cases. These data indicate that rM51R-M virus is at least as effective as rwt virus in reducing LNCaP tumors in vivo when administered intratumorally and support our hypothesis that M protein mutant viruses are safer viruses for anti-tumor therapies due to their ability to induce antiviral responses and spare normal cells. In fact, the M mutant virus is also as effective as rwt virus at killing prostate tumor xenografts when administered systemically (Fig. 7B), further supporting the data that this

virus is able to spread and replicate in vivo.

Figure 7: rwt and rM51R-M viruses effectively kill LNCaP tumors in vivo. LNCaP tumors were implanted in the flanks of nude mice and treated intratumorally (A) or intravenously (B) with rwt or rM51R-M viruses. Tumor volume was measured and is expressed as a % of day 0. (C) Hematoxalin- and eosin-stained section of tumors harvested at 14 days post-treatment.

Histological examination of tumors by hematoxylin and eosin (H&E) staining at day 14 postinfection showed that mock-infected tumor cells had well defined cell borders and hyperchromatic nuclei (Fig. 7C). The cytoplasm of these cells was vesicular and eosinophilic, with evidence of mitoses. In contrast, the tumors treated with rwt and rM51R-M viruses were extensively necrotic, characterized by loss of nuclear staining, increased cytoplasmic eosinophilia, and loss of cellular detail and cell borders. The additional tissue eosinophilia in tumors infected with rM51R-M virus was due to infiltrating erythrocytes as a result of hemorrhaging. These data support the idea that the remaining tumor mass at day 14 consisted largely of dead or dying tumor cells.

Figure 8: Systemic Treatment of PC3 and LNCaP Tumors with Recombinant VSVs

Differential susceptibility of prostate cancers to oncolytic VSV. One might expect that individual tumors would vary in their susceptibility to treatment with oncolytic viruses, depending on the extent to which they downregulate their antiviral pathways during tumor development. Indeed, we have found that in contrast to LNCaP tumors, tumors derived from another human prostate cancer cell line, PC3, are resistant to treatment with either rwt or rM51R-M viruses. These data are shown in Figure 8. In this series of experiments, nude mice bearing subcutaneous tumors derived from either LNCaP or PC3 cells were treated systemically by intravenous injection of either rwt or rM51R-M viruses. The LNCaP-derived tumors regressed with a time course similar to that resulting from intratumoral treatment. However, the PC3-derived tumors continued to grow, similar to mock-treated controls.

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Immunohistochemical analysis of tumors after two weeks showed the presence of viral antigen-positive cells in the PC3-derived tumors, but they were far fewer than in LNCaP-derived tumors (Ahmed, Cramer, and Lyles, 2004).

Key research accomplishments:

1. M protein mutant viruses are effective killers of prostate tumor cells.
2. M protein mutant viruses effectively induce interferon in normal prostate cells and tumor cells.
3. LNCaP and PC-3 prostate tumor cells are differentially responsive to interferon. LNCaP tumor cells are not responsive to interferon, while PC-3 cells are responsive.
4. We have identified an M protein mutant virus, rM51R, as a potential vector for tumor therapy. This virus effectively kills tumor cells and induces high levels of interferon in normal prostate cells.
5. Both rwt and rM51R viruses are effective killers of LNCaP tumors in nude mice. However, most of the mice treated with rwt virus succumb to virus infection, while mice treated with rM51R-M virus show no signs of virus-induced illness.
6. The difference in cell killing and responsiveness to interferon between LNCaP and PC-3 cells is reflected in their relative sensitivity (LNCaP) and resistance (PC3) to VSV treatment in nude mice.
7. In future experiments, the differences between LNCaP and PC3 cells will be exploited to determine which naturally-occurring tumors are likely to be responsive to oncolytic virus therapy with VSV.

Reportable outcomes:

1. Most of the results generated by this project were published in our manuscript in *Virology* (Ahmed, Cramer, and Lyles, 2004).
2. These results have been presented at the annual meeting of the American Society for Virology by Dr. Maryam Ahmed (Coinvestigator) in 2002, 2003, and 2004.

Conclusions:

Aim 1 of this proposal was to identify mutations in VSV genes that enhance the differential killing of prostate tumor cells versus normal cells. The results from our study indicated that the M protein mutant viruses effectively killed prostate cells *in vitro*. LNCaP tumor cells and benign prostatic cells were similarly sensitive to virus infection in single-cycle infection experiments, indicating that normal prostatic cells are not inherently resistant to virus infection. In multiple-cycle infection experiments, benign prostatic cells were resistant to infection with the M protein mutant virus (but not wild-type virus), due to the induction of antiviral responses by the mutant virus. In contrast, LNCaP tumor cells were effectively killed by the M protein mutant virus in both single-cycle and multiple-cycle experiments, due to their defective antiviral responses. These results support our basic hypothesis that M protein mutant viruses will show a greater selectivity for killing tumor cells versus normal cells. Furthermore, in Aim 1 we identified a tumor cell line that is less susceptible to virus infection (PC-3). We are interested in the differences between the PC3 and LNCaP cell lines since identifying these differences may allow us to predict which prostate tumors are likely to respond to oncolytic virus therapy with VSV.

Aim 2 was to identify VSV mutants that enhance the antiviral interferon response in prostate cells. We found that the M protein mutant viruses induced interferon in both normal prostate cells and prostate tumor cells. In addition, PC-3 and LNCaP cells were differentially responsive to interferon. Because of the differences between these two cell types, it will become important to establish secondary cultures of prostate tumors to determine which tumor cells line resembles prostate tumors in patients. Aim 3 was to determine whether VSV mutants have greater efficacy and safety than wild-type VSV in reducing prostate tumors in nude mice. Both rwt and

rM51R viruses are effective killers of LNCaP tumors in nude mice. However, most of the animals treated with rwt virus also succumbed to virus infection within 4 weeks post-treatment. In contrast, animals treated with rM51R virus eliminated the tumor and remained healthy for 2 months post-treatment.

The animal work in Aim 3 suggests that our M protein viruses can be used for effective and safe tumor therapies in humans. However, the differences in sensitivity between LNCaP and PC3 tumors suggest that we may need to screen individual tumors for susceptibility to oncolytic VSV treatment. These results support further testing of VSV M protein mutants in primates, and clinical trials within 5 years. There are compelling reasons for developing new treatments for prostate cancer, including the development of viral vectors. Approximately 1 out of every 10 men will develop this form of cancer, and it is second only to lung cancer as the leading cause of cancer death in men. Each year more than 30,000 deaths are caused by this disease. Current treatment of tumors that remain confined to the prostate gland is usually successful, with a 5-year survival rate of 88%. The challenge to develop novel therapies for prostate tumors is in the treatment of metastatic tumors that have spread to many other sites in the body or to areas that are difficult to access. The 5-year survival rate for patients with metastatic prostate cancer is only 29%. The use of viruses that have been genetically engineered to kill tumor cells offers a promising approach to the treatment of metastatic cancer, because of the natural ability of viruses to spread throughout the body and seek out the tissues that are susceptible to infection. Our mutant viruses have great potential to be used in such therapies due to their added features of safety and of targeting tumor cells due to inherent cellular defects in the interferon pathway.

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APPENDIX

Ahmed, M., Cramer, S. D., and Lyles, D. S. (2004). Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. *Virology* **330**(1), 34-49.

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Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses

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Abstract

Because of its potent ability to induce apoptosis, vesicular stomatitis virus (VSV) is an attractive candidate as an oncolytic virus for tumor therapy. Previous studies have suggested that VSV selectively infects tumor cells due to defects in their antiviral responses making them more susceptible to VSV infection than normal cells. We tested this hypothesis in the prostate tumor system by comparing LNCaP and PC-3 prostate tumor cells to benign human prostatic epithelial cells from patient prostatectomy specimens. We compared the cell killing ability of a recombinant virus containing a wild-type (wt) M protein (rwt) and an isogenic M protein mutant virus (rM51R-M) that induces interferon (IFN) in infected cells and should display a greater selectivity for tumor cells. Our results showed that in single-cycle infection experiments, LNCaP cells were sensitive to killing by both wt and mutant viruses, while PC-3 cells were highly resistant to VSV-induced cell killing. LNCaP and benign prostate cells were similarly susceptible to both viruses, indicating that normal prostate cells are not inherently resistant to killing by VSV. In each of the cell lines, the rM51R-M virus induced similar levels of apoptosis to rwt virus, showing that the M protein does not play a significant role in apoptosis induction by VSV in these cells. In multiple-cycle infection experiments, LNCaP cells were more sensitive than benign prostatic epithelial cells to virus-induced cell killing by rM51R-M virus, but not rwt virus. Both viruses were equally effective at reducing LNCaP tumor volume in vivo following intratumoral and intravenous inoculation in nude mice, while PC-3 tumors were resistant to VSV treatment. None of the mice treated with rM51R-M virus died as a result of virus infection, while 50–71% of mice treated with rwt virus succumbed to virus infection. Similarly, when inoculated by the more sensitive intranasal route, the rM51R-M virus was less pathogenic than the rwt virus from which it was derived. These results indicate that M protein mutant viruses are superior candidates as oncolytic viruses for therapies of prostate tumors, but future strategies for use of VSV will require testing individual tumors for their susceptibility to virus infection.
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Keywords: Vesicular stomatitis virus; Prostate tumor; Interferon; Matrix protein

Introduction

Cytocidal viruses are currently being explored as onco-lytic agents for anti-tumor therapies (reviewed by Bell et al., 2003; Giedlin et al., 2003). Vesicular stomatitis virus (VSV) is an attractive candidate as an oncolytic agent because it is a potent inducer of apoptosis in infected cells (Balachandran et al., 2000; Kopecky et al., 2001; Koyama, 1995), and many

types of tumor cells are susceptible to infection and killing by VSV (Balachandran and Barber, 2000, 2001; Stojdl et al., 2000, 2003). It has been proposed that the susceptibility of tumors to VSV and other oncolytic viruses is due to development of defects in antiviral responses during tumori-genesis (Balachandran and Barber, 2000, 2001; Bell et al., 2003; Stojdl et al., 2000, 2003). This may occur because in many cases, proliferative signaling pathways and antiviral signaling pathways are mutually antagonistic. Thus, develop-ment of enhanced proliferative signaling in tumor cells may lead to suppression of antiviral pathways. For example, it has been known for many years that most tumor cells are resistant to the antiproliferative effects of interferons (IFNs) (Einhorn

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and Strander, 1993). In fact, several different defects in the IFN signal transduction pathway have been identified in different types of tumors (reviewed in Wong et al., 1997). The resistance of tumor cells to the antiproliferative effects of IFNs may make these cells correspondingly more susceptible to infection with a variety of viruses, including VSV (Balachandran and Barber, 2000, 2001; Stojdl et al., 2000). However, it is also possible for tumors to develop resistance to the antiproliferative effects of IFN without developing defects in their antiviral responses (Pfeffer et al., 1996).

The idea that cells will develop defects in their antiviral responses during tumorigenesis is a fundamental principle of oncolytic virus therapy. However, there are very few examples in which the susceptibility of tumor cells to virus-induced killing has been compared directly with that of normal cells from which they were derived. We chose to address this question using human prostate tumors for two important reasons. First, prostate cancer is the most frequently diagnosed cancer in men and the second leading cause of cancer-related deaths in the USA (Gotoh et al., 2003; Polek et al., 2003). No effective therapy is available for treating prostate cancer once the tumor metastasizes from the prostate during the course of the disease. Therefore, the development of novel and effective nonsurgical interventions, such as oncolytic viral therapies, for prostate cancer would represent a significant advantage for managing patients with metastatic prostate cancer. Secondly, prostatic epithelial cells derived from benign epithelium can be cultured from tissues obtained from patients, so that we can assess the effects of VSV infection on benign cells from the prostate and compare results to those in prostate tumor cell lines.

If tumor cells are defective in their antiviral responses, then the selectivity of viruses for tumor cells versus normal cells can be enhanced by strategies that activate antiviral responses in normal cells. Indeed, it has been shown that the selectivity of VSV for tumor cells versus normal cells can be enhanced either by pretreatment with IFNs or by using VSV strains that induce IFN production in infected cells (Obuchi et al., 2003; Stojdl et al., 2000, 2003). Most wild-type (wt) strains of VSV induce relatively little IFN production in infected cells. We have shown that this is due to the inhibition of host gene expression by the viral matrix (M) protein (Ahmed et al., 2003). The VSV M protein is a multifunctional protein that plays a major role in virus assembly as well as in the inhibition of host gene expression. The inhibition of host gene expression occurs at multiple levels, including transcription, nuclear-cytoplasmic RNA transport, and translation (reviewed in Lyles, 2000). This inhibition suppresses the production of IFNs and other antiviral proteins in infected cells (Ahmed et al., 2003; Stojdl et al., 2003).

The inhibitory effects of M protein on host gene expression are genetically separable from its virus assembly functions, so that a number of different mutations render the M protein defective in its ability to inhibit host gene expression without compromising its ability to function in virus assembly (Ahmed and Lyles, 1997; Ahmed et al., 2003;

Black et al., 1993). Viruses containing such M protein mutations induce IFN production in infected cells (Ahmed et al., 2003; Francoeur et al., 1987; Marcus et al., 1993; Stojdl et al., 2003). For the experiments presented here, we compared a recombinant virus obtained from an infectious cDNA clone containing a wt M protein (rwt virus) to an isogenic recombinant containing a single point mutation that substitutes an arginine for methionine at position 51 of the 229 amino acid M protein (rM51R-M virus). This point mutation renders the virus defective in its ability to inhibit host gene expression, but does not compromise the expression of viral genes or the production of infectious progeny (Ahmed et al., 2003; Kopecky et al., 2001). According to the basic principle of oncolytic virus therapy, normal prostatic epithelial cells should be resistant to the M protein mutant virus due to activation of antiviral responses, while tumor cells should be susceptible due to defects in their antiviral responses.

In the experiments presented here, we found that one line of prostate tumor cells (LNCaP) was susceptible to killing with VSV, while another line (PC-3) was more resistant, suggesting that treatment of prostate tumors with oncolytic VSV will be effective for some, but not all tumors. Interestingly, benign prostatic epithelial cells were similar to LNCaP cells in their susceptibility to both wt and M protein mutant VSV in single-cycle infection experiments. These results show that normal prostatic epithelial cells are not inherently resistant to VSV. Under multiple cycle infection conditions, benign prostatic cells were resistant to rM51R-M virus, but not the rwt virus control, while LNCaP cells were susceptible to both viruses. Thus, activation of antiviral responses in normal cells by the M protein mutant virus can prevent virus spread to surrounding uninfected cells. Similar to cell culture results, LNCaP cells implanted in the flanks of nude mice were equally susceptible to rwt and rM51R-M viruses, with complete tumor regression in some cases. However, while greater than 50% of mice treated with the rwt virus died due to virus infection, all of the mice treated either intratumorally or intravenously with rM51R-M virus remained healthy. Likewise, the rM51R-M virus was less pathogenic for mice than rwt virus when assayed using the more sensitive route of intranasal inoculation. These results indicate that M protein mutant viruses are promising candidates as oncolytic viruses because of their ability to spread systemically without causing disease symptoms, and are safer vectors for tumor therapies due to their ability to effectively kill tumor cells, while sparing normal tissue.

Results

LNCaP and PC-3 prostate cells are differentially responsive to IFN

Clinical studies indicate that metastatic prostate tumors are not responsive to IFN treatments (DiPaola and Aisner, 1999; Tjoa and Murphy, 2000). Therefore, prostate tumors should

be correspondingly susceptible to VSV infection. However, our results indicate that tumor cells derived from the same tissue vary in their responsiveness to IFN. Fig. 1 illustrates the differential responsiveness of prostate tumor cells to IFN
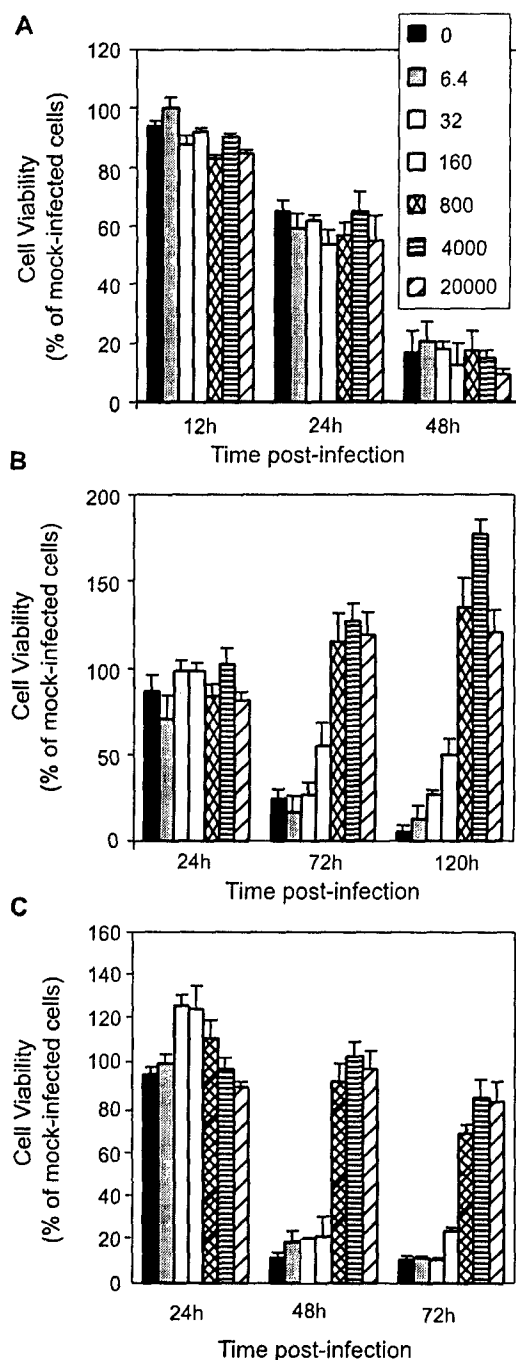

Fig. 1. Responsiveness of LNCaP and PC-3 cells to IFN. LNCaP (A), PC-3 (B), and HeLa (C) cells were incubated with varying concentrations of IFN (6.4–20000 IU/ml) for 16 h and challenged with wt VSV at an MOI of 0.015 pfu/cell (LNCaP and HeLa cells) or 30 pfu/cell (PC-3 cells). At various times postinfection, cell viability was measured by MTT assay. Controls included IFN-untreated cells infected with VSV- and IFN-treated cells that were not challenged with VSV. Data are expressed as a percentage of mock-infected cells and are the averages of two separate experiments.
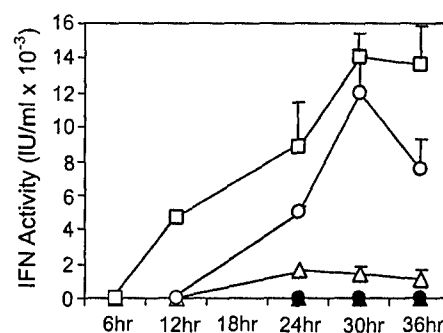
Fig. 2. Interferon bioactivity produced by cells infected with wt and M protein mutant viruses. LNCaP (triangles), PC-3 (squares), and normal prostatic (circles) cells were infected with wt (closed symbols) and rM51R-M (open symbols) viruses at a multiplicity of 10 pfu/cell for the indicated times. Supernatants were collected and residual virus was inactivated by incubation at pH2. HeLa cells were incubated overnight with serial dilutions of supernatants and were challenged with wt VSV. Cells were incubated overnight, then fixed and stained with crystal violet. Absorbance was read at 550 nm on an ELISA reader. The interferon concentration (IU/ml) was quantitated by comparing results to those in cells incubated with serial 5-fold dilutions of an interferon standard. Data shown are means for two independent experiments.

using LNCaP and PC-3 cell lines, both of which are derived from metastatic prostate tumors. LNCaP and PC-3 cells were seeded onto 96-well dishes and incubated with varying concentrations of IFN. After 16 h, cells were challenged with VSV at an MOI of 0.015 pfu/cell for LNCaP cells and an MOI of 30 pfu/cell for PC-3 cells. The different MOIs used for the challenge reflect the doses of VSV required to induce cytopathic effect in each of the cell lines. At different times postinfection, cell viability was measured by MTT assay. Cells responsive to IFN should exhibit an IFN dose-dependent resistance to virus infection, while cells that are not responsive to IFN should show no difference in cytopathic effect regardless of IFN concentration. HeLa cells were used as control since they are known to respond to IFN. Data in Fig. 1A show a decrease in LNCaP cell viability over the time course of the experiment due to virus infection. Furthermore, the results indicate that LNCaP cells are not responsive to IFN since they were not protected from VSV infection by pretreatment with IFN concentrations as high as 20000 IU/ml. In contrast, PC-3 cells were responsive to IFN (Fig. 1B) since close to 100% of cells displayed resistance to VSV infection upon pretreatment with concentrations of IFN ≥800 IU/ml. The sensitivity of PC-3 cells to IFN was similar to that observed with HeLa cells (Fig. 1C). These results indicate that prostate tumor cells can differ dramatically in their response to IFN and suggest that they may also be different in their sensitivity to oncolytic VSV.

We have previously generated recombinant M protein mutants of VSV that are defective in their ability to suppress IFN production by infected cells (Ahmed et al., 2003). Our original hypothesis was that these mutant viruses would be defective in their ability to replicate in normal cells or in IFN-responsive tumor cells like PC-3 cells, but should replicate well in tumor cells that have defects in their IFN responses,

such as LNCaP cells. In order to test this hypothesis, we first determined the ability of benign prostate epithelial cells compared to prostate tumor cells to produce IFN in response to infection with isogenic recombinant viruses containing wt (rwt virus) or mutant M protein (rM51R-M virus).

Primary cultures of normal human prostate epithelial cells were obtained from a benign region of the prostate peripheral

zone of a patient prostatectomy specimen (WFU55PZ strain). These cultures have been extensively characterized to be representative of normal human epithelial cells as described in Materials and methods. WFU55PZ cells, LNCaP cells, or PC-3 cells were infected with either rwt or rM51R-M virus, and IFN released into the media was assayed at varying times postinfection. IFN levels were determined by a
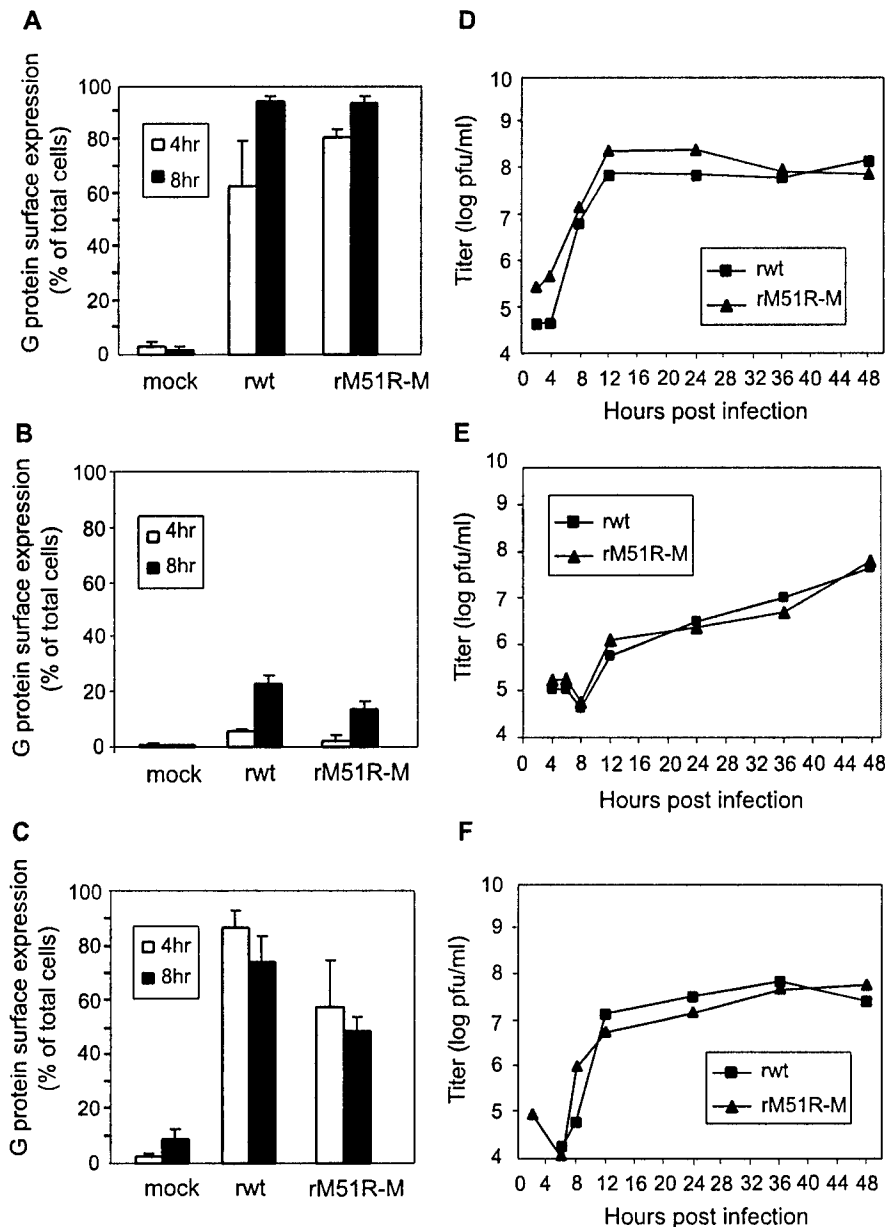
Fig. 3. Single-cycle infection of prostate tumor and benign cells with wt and M protein mutant viruses. G protein surface expression in LNCaP (A), PC-3 (B), and WFU55PZ benign prostate (C) cells infected with rwt and rM51R-M viruses. The efficiency of G protein cell surface expression during VSV infection was determined by flow cytometry analysis of LNCaP, PC-3, and normal prostate cells infected with wt and mutant viruses. Cells were infected with rwt and rM51R-M viruses at a multiplicity of 10 pfu/cell for 4 and 8 h. After infection, cells were labeled with anti-G protein antibody followed by a secondary antibody conjugated to fluorescein. G protein cell surface fluorescence was quantitated with a flow cytometer. The surface expression of G protein is shown as a percentage of total cells with fluorescence above the negative control mock-infected cells. Data shown are the mean ± SEM of four independent experiments. Single-cycle virus growth analysis in LNCaP (D), PC-3 (E), and WFU55PZ (F) cells. Cells were infected with wt and mutant viruses at a multiplicity of 10 pfu/cell. A small aliquot of the supernatant was removed at the indicated times postinfection to determine the amount of progeny virus by plaque assay. Data are the average of two independent experiments.

bioassay based on reduction of VSV cytopathic effect in HeLa cells in the presence of supernatants from infected prostate cells (Fig. 2). Infection with rM51R-M virus induced IFN production in all three cell types (open symbols). In contrast, no IFN bioactivity was detectable in cells infected with rwt virus (closed symbols). These results are consistent with earlier data showing that M protein mutant viruses induce IFN production, while viruses with wt M protein suppress IFN production by infected cells. Although both benign cells (open circles) and PC-3 cells (open squares) produced substantial levels of IFN, PC-3 cells actually produced slightly higher levels of IFN than benign prostate cells, indicating that the production of IFN in tumor cells can actually be greater than that in the normal cells from which they were derived. LNCaP cells produced low, but detectable levels of IFN following infection with rM51R-M virus (open triangles). Collectively, the results in Figs. 1 and 2 with LNCaP cells are generally consistent with the idea that tumor cells have less effective antiviral responses than corresponding normal cells, but the results with PC3 cells show that prostate tumor cells can differ dramatically in their antiviral responses.

PC-3 cells are more resistant than LNCaP and benign prostate cells to infection with wt and mutant viruses under single-cycle infection conditions

To determine whether the sensitivity of tumor and benign prostate cells to VSV infection correlates with their IFN responses, we tested the ability of rwt and rM51R-M viruses to infect LNCaP and PC-3 tumor cells as well as benign prostate cells in single-cycle infection experiments. Cells were infected at a multiplicity of 10 pfu/cell, so that in theory, nearly 100% of cells were infected. The efficiency of infection was measured by determining the percentage of cells expressing the viral G protein surface antigen by flow cytometry at 4 and 8 h postinfection, and by determining yields of progeny virus by plaque assay over a 48 h time course (Fig. 3). As expected from the low levels of their antiviral responses, LNCaP cells were highly susceptible to VSV infection, with nearly 100% of cells expressing G protein at the cell surface by 8 h postinfection, and production of progeny virus at approximately 10^8 pfu/ml by 12 h postinfection (Figs. 3A and D). There was little difference between rwt and rM51R-M viruses in their ability to produce infectious progeny in LNCaP cells, with the M protein mutant virus producing slightly higher titers than its isogenic wt control. This result is consistent with our earlier data in other cell types showing that the M51R M protein mutation has little effect on the virus assembly function of M protein, and that the rM51R-M virus produces slightly higher virus yields than rwt virus (Ahmed et al., 2003).

In contrast to LNCaP cells, infection of PC-3 cells with VSV was much less efficient, with only 10–20% of cells expressing G protein above background levels by 8 h postinfection, and production of lower levels of progeny virus

over a longer time course (Figs. 3B and E). It appeared that a slightly lower percentage of PC-3 cells infected with rM51R-M virus expressed G protein on their surface than cells infected with rwt virus. However, the yields of infectious progeny of the two viruses were nearly indistinguishable, despite the fact that the M protein mutant virus induced
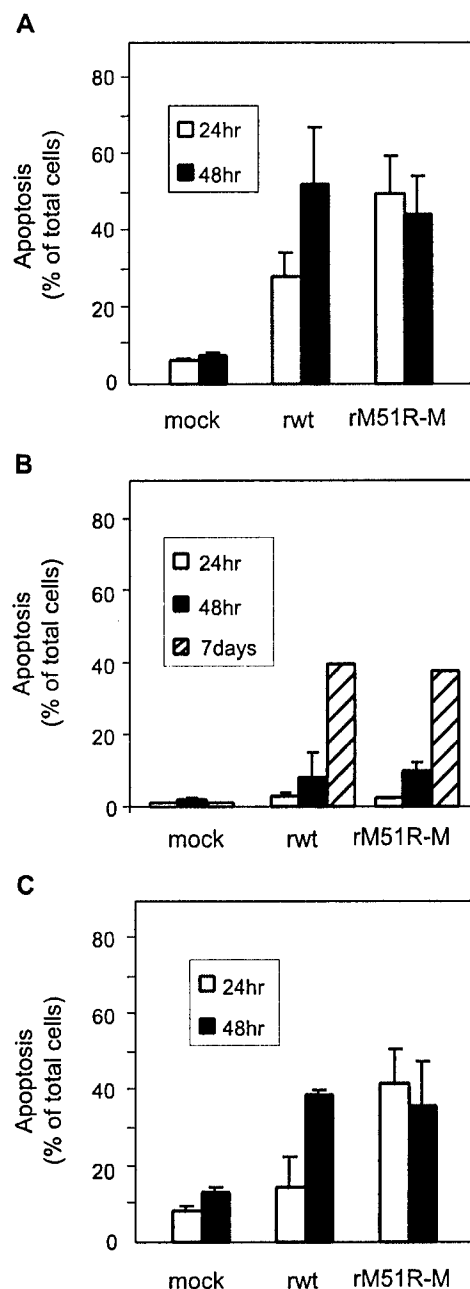

Fig. 4. Induction of DNA fragmentation in LNCaP (A), PC-3 (B), and WFU55PZ (C) cells infected with rwt and rM51R-M viruses. Cells were infected with wt and mutant viruses at a multiplicity of 10 pfu/cell. At 24 and 48 h postinfection, cells were harvested, fixed, and permeabilized. Cells were incubated with the TUNEL reaction mixture according to the manufacturer's directions, and the incorporated fluorescein was analyzed and quantitated by flow cytometry. Data are expressed as the percentage of total cells undergoing apoptosis and are the means ± SD of four experiments.

IFN production in these cells, while the virus wt M protein did not (Fig. 2). These results suggest that the relative resistance of PC-3 cells to VSV infection under single-cycle conditions is due to antiviral factors in addition to IFN responses.

To our surprise, the results obtained with benign prostate cells under single-cycle infection conditions were more similar to those in LNCaP cells than PC-3 cells. Seventy to ninety percent of WFU55PZ cells infected with the rwt virus expressed the viral G protein (Fig. 3C). Cells infected with rM51R-M virus expressed G protein to a slightly lesser extent (50–60% of cells) than cells infected with rwt virus, although this difference was not statistically significant. Likewise, there was little if any difference between rwt and rM51R-M viruses in yield of infectious progeny from WFU55PZ cells (Fig. 3F). The time course of virus production by WFU55PZ cells was intermediate between those of LNCaP and PC-3 cells. These results show that the generalization that tumor cells are more susceptible to virus infection than normal cells is not always true. In the case of the prostate, normal epithelial cells are quite susceptible to VSV infection. Some tumor cells, such as LNCaP, are even more susceptible, while others, such as PC-3 cells, are more resistant.

M protein mutation has little effect on VSV-induced apoptosis in prostate cells

A crucial question for tumor therapy is whether M protein mutant viruses induce apoptosis in infected prostate cells, since M protein mutants are delayed in their induction of apoptosis in some cell types (Kopecky et al., 2001). Furthermore, it is important to determine whether prostate cancer cells are differentially susceptible to apoptosis induction by VSV. Therefore, the percentage of cells undergoing DNA fragmentation characteristic of apoptotic cells was determined by TUNEL assay. LNCaP, PC-3, and WFU55PZ cells were infected with rwt and rM51R-M viruses at an MOI of 10 pfu/cell. At 24 and 48 h postinfection, cells were fixed, permeabilized, and incubated with a TUNEL reaction mixture containing a fluorescent label, and the percentage of cells containing DNA strand breaks was determined by flow cytometry. At 24 h postinfection, a higher percentage of LNCaP cells infected with the rM51R-M virus was apoptotic as compared to cells infected with the rwt virus (Fig. 4A, white bars). However, at 48 h postinfection, there was no difference between cells infected with wt and rM51R-M viruses (black bars). In contrast to LNCaP cells, a lower percentage of PC-3 cells (5–10%) infected with wt and mutant viruses was apoptotic by 48 h postinfection (Fig. 4B). However, there was detectable apoptosis at day 7 postinfection (hatched bars). WFU55PZ cells were also susceptible to apoptosis induced by both wt and mutant viruses (Fig. 4C), but not to the same degree as LNCaP cells. These results indicate that LNCaP and PC-3 prostate tumor cells vary in their sensitivity to VSV-induced apoptosis. However, the M protein mutant virus was as
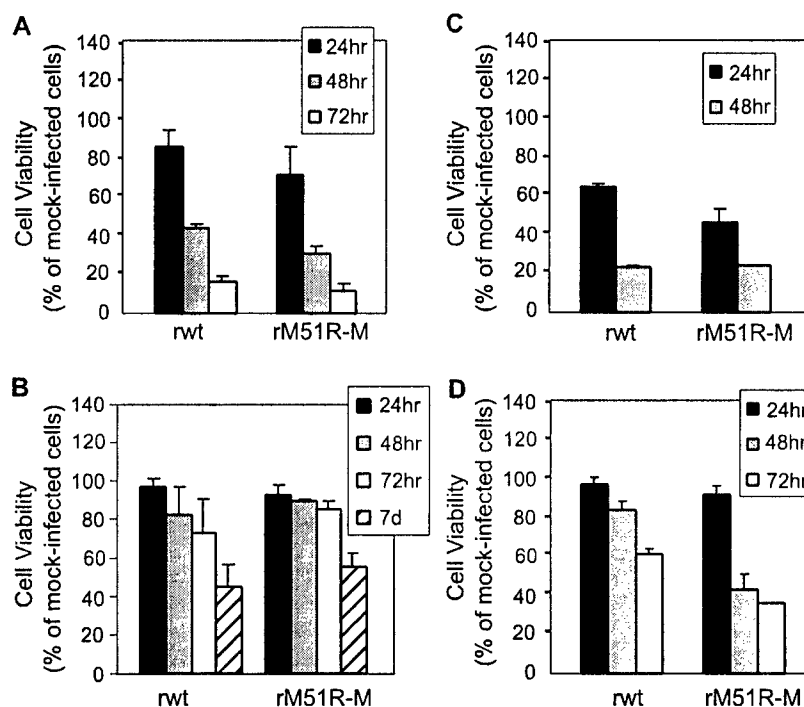

Fig. 5. Cell viability of prostate tumor and benign cells infected with wt and mutant viruses under single-cycle infection conditions. LNCaP (A), PC-3 (B), WFU55PZ (C), and WFU232BPH (D) cells were infected with wt and mutant viruses at a multiplicity of 10 pfu/cell. At the indicated times postinfection, live cells were measured by MTT assay. Data are expressed as a percentage of the cell viability of mock infected cells and represent the means ± SD of three or four experiments.

effective as the rwt virus in the induction of apoptosis in prostate cells. Thus, M protein plays little if any role in the induction of apoptosis in these cells.

The ability of wt and M protein mutant viruses to kill prostate tumor and benign cells was confirmed by MTT assay. These experiments compared LNCaP, PC-3, and WFU55PZ cells, and another benign prostate cell strain isolated from a patient with benign prostatic hyperplasia, WFU232BPH. Cells were infected at an MOI of 10 pfu/cell with rwt and rM51R-M viruses, and cell viability was measured at different times postinfection (Fig. 5). Results show that LNCaP cells were sensitive to killing by wt and mutant viruses and that there was no difference between wt and mutant viruses in their ability to kill these cells (Fig. 5A). Consistent with DNA fragmentation data shown in Fig. 4, PC-3 cells were

resistant to killing by VSV (Fig. 5B). However, viability of cells infected with wt and mutant viruses decreased by day 7 postinfection. WFU55PZ benign prostatic epithelial cells were sensitive to killing by each of the viruses so that by 48 h postinfection, approximately 20% of infected cells remained viable (Fig. 5C). These results were also consistent with apoptosis data obtained in Fig. 4C. WFU232BPH cells were somewhat more resistant to virus-induced cell killing, with approximately 40% of cells remaining viable at 72 h postinfection (Fig. 5D). Despite this difference between prostate cells from different patients, we can conclude from Figs. 3, 4, and 5 that LNCaP prostate tumor and benign cells are susceptible to infection and killing with wt and mutant viruses under single-cycle infection conditions, while PC-3 cells are more resistant. Furthermore, these results
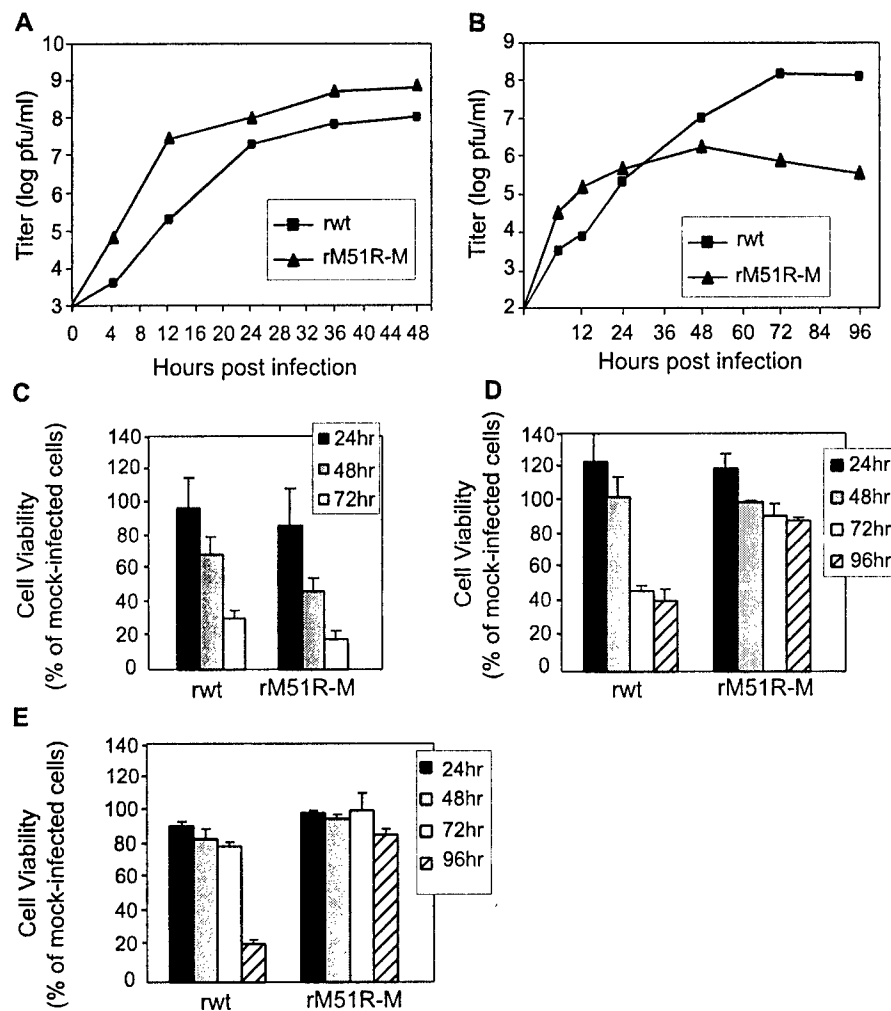

Fig. 6. Multiple cycle infection of LNCaP and benign prostate cells with wt and M protein mutant viruses. Multiple cycle growth analysis of virus in LNCaP (A) and WFU55PZ (B) cells. Cells were infected with wt and mutant viruses at a multiplicity of 0.1 pfu/cell. A small aliquot of the supernatant was removed at the indicated times postinfection to determine the amount of progeny virus by plaque assay. Data are the average of two independent experiments. Cell viability of LNCaP (C), WFU55PZ (D), and WFU232BPH (E) cells infected with wt and mutant viruses. Cells were infected at a multiplicity of 0.1 pfu/cell and cell viability was measured by an MTT assay. Data are expressed as a percentage of the cell viability of mock-infected cells and represent the means ± SD of three or four experiments.

indicate that the M protein does not play a significant role in VSV-induced apoptosis in the prostate.

Benign prostate cells are more resistant to infection with M51R-M virus than wt viruses in multiple-cycle infection experiments

To determine whether antiviral responses in infected cells affect spread of virus to surrounding uninfected cells, we tested the ability of wt and mutant viruses to infect LNCaP and benign prostate cells under multiple-cycle infection conditions (MOI = 0.1 pfu/cell). Similar to data obtained under single-cycle infection conditions, rM51R-M grew to titers slightly higher than those of rwt virus in LNCaP cells in the multiple-cycle growth experiment (Fig. 6A). Although both viruses reached titers as high as those observed in the single-cycle growth experiment (Fig. 3D), there was a delay in growth due to differences in kinetics of virus spread. This delay was also seen in WFU55PZ cells infected with each of the viruses (Fig. 6B). However, in contrast to LNCaP cells, the rM51R-M virus produced lower levels of infectious virus progeny than rwt virus in WFU55PZ cells. In fact, by 96 h postinfection, rM51R-M virus titers were 2 logs lower than those of rwt virus. Similar data were obtained from WFU232BPH benign prostate cells (data not shown). These data suggest that the elevated antiviral response in benign prostate cells infected with rM51R-M virus limits virus spread to surrounding cells.

The ability of wt and mutant viruses to kill LNCaP and benign prostate cells under multiple-cycle infection conditions was measured by MTT assay. Data indicate that LNCaP cells were equally susceptible to killing by both of the viruses (Fig. 6C). However, both isolates of benign prostate cells were highly resistant to cell killing by rM51R-M virus. Close to 90% of benign prostate cells infected with rM51R-M virus retained viability at 96 h postinfection (Figs. 6D and E). In contrast, both cell strains were sensitive to killing by rwt virus. As expected from single-cycle experiments, PC-3 cells remained resistant to killing by wt and mutant viruses under multiple-cycle infection conditions (data not shown). These results indicate that in contrast to data obtained in the single-cycle experiments, primary cultures of benign prostate cells are consistently resistant to infection with rM51R-M virus in multiple-cycle infection experiments, likely due to the ability of this virus to induce an effective antiviral response in these cells as compared to LNCaP cells.

Rwt and rM51R-M viruses effectively kill LNCaP tumor cells in vivo

To compare the ability of rwt and rM51R-M viruses to kill prostate tumor cells in vivo, LNCaP and PC-3 cells were injected subcutaneously in the flanks of BALB/c nude mice, and when palpable tumors were obtained, mice were treated with rwt and rM51R-M viruses. Tumor-bearing mice were
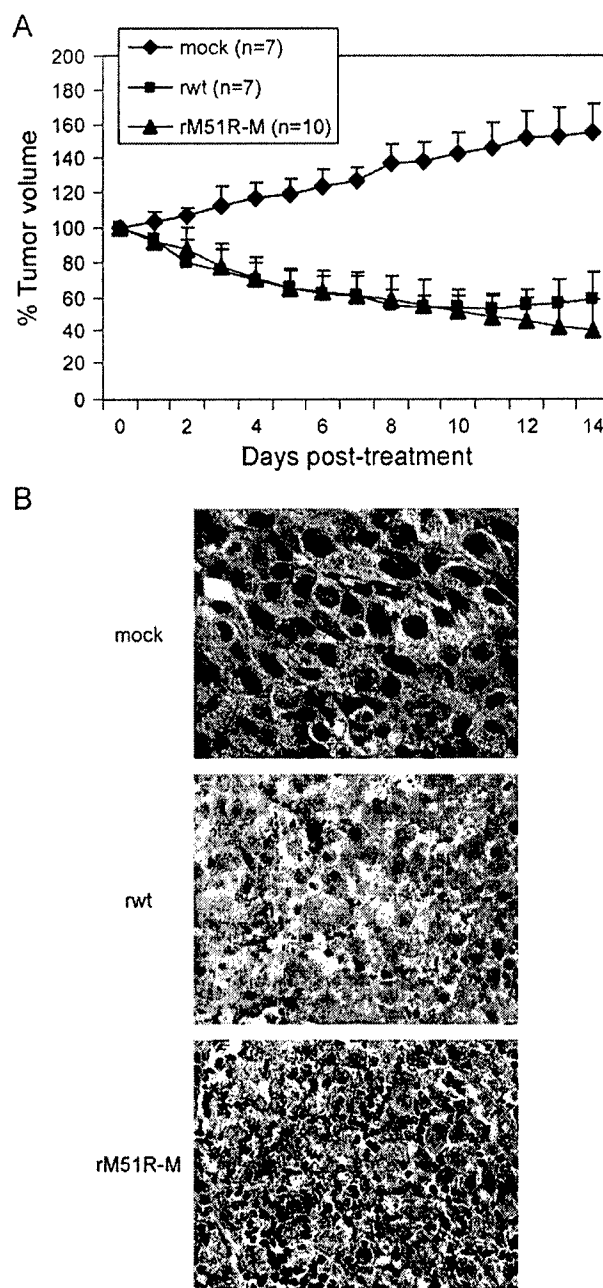
A

B

Fig. 7. Treatment of LNCaP tumors with rwt and rM51R-M viruses. LNCaP cells were injected subcutaneously in the flank of BALB/c nude mice. Animals with palpable tumors were randomly separated into three experimental groups (4–5 animals/group) and were injected in the tumor with 1 × 10^8 pfu of rwt (n = 7) or rM51R-M (n = 10) viruses in culture medium or with culture medium alone (n = 7) as a negative control. Tumor volume was measured daily with calipers. Animals showing symptoms of end-stage VSV infection or signs of terminal illness were sacrificed and the tumor and selected tissues (brain, lungs, spleen, and liver) were harvested for histology. (A) Data represent three separate experiments and are shown as the percentage of original tumor size on day 0 (mean ± SD). (B) Histological examination of tumors by routine hematoxylin and eosin staining.

injected either intratumorally (Fig. 7A) or intravenously (Fig. 8A), and tumor volume was measured daily. LNCaP tumor volume decreased in animals treated intratumorally with both rwt and rM51R viruses, while mock-treated tumors continued to increase in size (Fig. 7A). By 2 weeks posttreatment, the tumors of mice injected with rM51R-M virus had decreased to approximately 40% of their initial volume at the start of the experiment. In fact, after day 14, the percent change in volume of tumors treated with rM51R-M virus (mean percent decrease = 60.6) was significantly lower than rwt virus (mean percent decrease 37.4) treated tumors (P = 0.048). Interestingly, although the tumors of mice treated with rwt virus also regressed, some tumors started to increase in size again by 10 days posttreatment. Statistical analysis indicated that tumors treated with rwt virus did not decrease further between days 7 and 14 (P = 0.68), whereas tumors treated with

rM51R-M virus significantly decreased (P = 0.0014) during that time.

Histological examination of tumors by hematoxylin and eosin (H and E) staining at day 14 postinfection showed that mock-infected tumor cells had well-defined cell borders and hyperchromatic nuclei (Fig. 7B). The cytoplasm of these cells was vesicular and eosinophilic, with evidence of mitoses. In contrast, tumors treated with rwt and rM51R-M viruses were extensively necrotic, characterized by loss of nuclear staining, increased cytoplasmic eosinophilia, and loss of cellular detail and cell borders. The additional tissue eosinophilia in tumors infected with rM51R-M virus was due to infiltrating erythrocytes as a result of hemorrhaging. These data support the idea that the remaining tumor mass at day 14 consisted largely of dead or dying tumor cells.

Results similar to those obtained in Fig. 7A were observed when LNCaP tumor-bearing animals were sys-
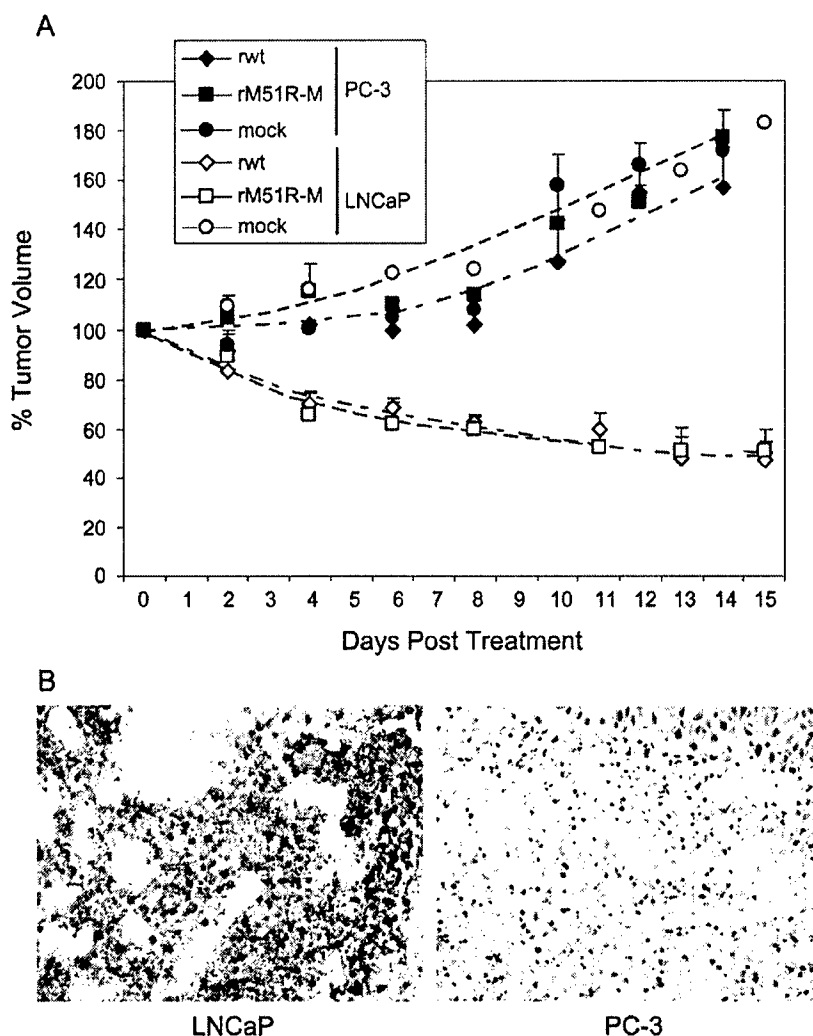

Fig. 8. Tumors derived from PC-3 cells are resistant to treatment with wt and mutant viruses. (A) LNCaP and PC-3 cells were injected subcutaneously in the flank of BALB/c nude mice. When palpable tumors had formed, animals were injected intravenously with 2 × 10^7 pfu of rwt or rM51R-M viruses in culture medium or with culture medium alone as a negative control. Four animals per group were used for LNCaP tumor experiments, and two animals per group were used for PC-3 tumor experiments. Data are shown as the percentage of original tumor size on day 0 (mean ± SD). (B) Viral antigen staining in sections of LNCaP and PC-3 tumors from mice treated with rwt virus.

temically treated with wt and mutant viruses (Fig. 8A, open symbols). By day 7 posttreatment, tumors of mice injected with each of the viruses decreased to approximately 60% of their initial volume, and further decreased to approximately 50% of their initial volume by 2 weeks posttreatment. As expected from the results of tissue culture experiments, PC-3 tumors (closed symbols) were highly resistant to treatment with either rwt or rM51R-M virus and continued growing in size at a rate similar to mock-treated tumors. Immunohistochemical analysis of tumors from mice at day 14 (PC-3 tumors) and day 15 (LNCaP tumors) posttreatment was carried out with antibodies against the viral G protein to determine the ability of each of the viruses to replicate and spread in the tumor tissue (Fig. 8B). Viral antigen was detected at high levels in LNCaP-derived tumors, with evidence of extensive cell killing. In contrast, much fewer cells were positive for viral antigen in PC-3 tumors, and the cells remained healthy. These data further support the idea that prostate tumors vary in their sensitivity to treatment with VSV.

The M protein mutant virus is less pathogenic than viruses with wt M proteins

Although there was little if any difference between rM51R-M and rwt viruses in their effects on tumors, there was a striking difference in the morbidity of treated mice. Seventy-one percent of mice treated intratumorally and 50% of mice treated systemically with the rwt virus showed severe symptoms of VSV infection, including paralysis, weight loss, and disorientation, ultimately leading to death between days 7 and 14. However, none of the mice treated with rM51R-M virus showed signs of illness and there was complete elimination of tumors by 6–8 weeks in some cases.

To confirm differences in pathogenesis exhibited between rwt and rM51R-M viruses following treatment, 5-week-old male BALB/c mice were inoculated with varying doses of these viruses by the intranasal route, which is a more sensitive indicator of viral pathogenesis. Studies have shown that VSV can enter the brain through the olfactory tract, resulting in an acute infection of the CNS (Huneycutt et al., 1993, 1994). Mice were monitored daily for hind-limb paralysis, weight loss, and other signs of illness. Mice showing signs of terminal illness were euthanized and scored as nonsurvivors according to AAALAC recommendations. Organs were collected for immunohistochemistry and plaque assay to determine spread of virus. As a control for these experiments, we included a naturally occurring wt strain of VSV (Orsay strain) since earlier data had suggested that recombinant strains of VSV might be less pathogenic than naturally occurring strains.

Illness was observed in mice infected with both wtO and rwt viruses. The LD_{50} of the wtO strain was between 10^3 and 10^4 pfu (Fig. 9A). The percent survival of mice inoculated with rwt virus was significantly higher than mice infected with wtO virus ($P = 0.021$) with a LD_{50}

between 10^5 and 10^6 pfu. Interestingly, few mice infected with rwt virus displayed signs of hind-limb paralysis. Instead, these mice exhibited other signs of illness such as weight loss, decreased locomotion, and disorientation, ultimately leading to death. In addition, mice infected with rwt virus succumbed to infection at least 2–3 days after the mice that were infected with wtO virus (data not shown). In contrast, all mice infected with rM51R-M virus, with doses
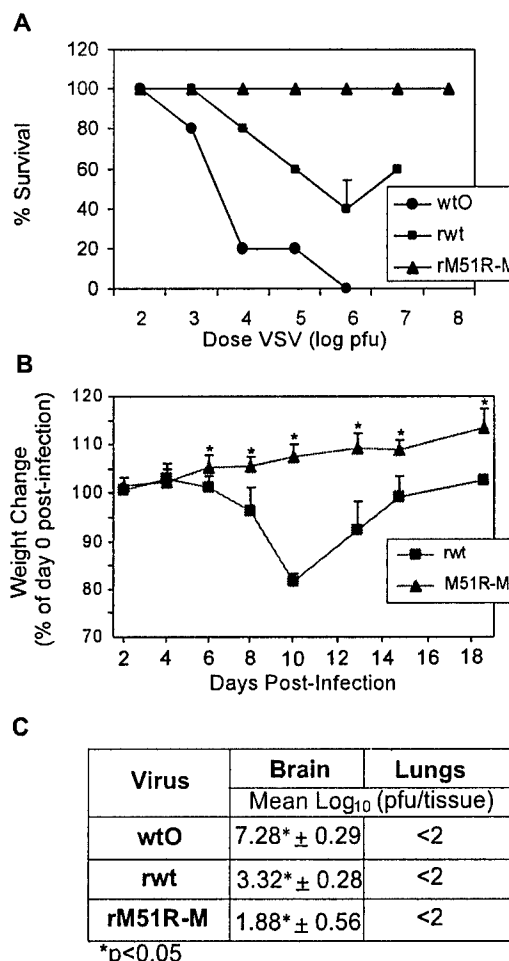

Fig. 9. Determination of the median lethal dose (LD_{50}) for BALB/c mice infected with wt and M protein mutant viruses. Six-week-old BALB/c male mice (Charles River) were intranasally infected with the indicated titers of wtO, rwt, and rM51R-M viruses (10^2–10^8 pfu/mouse) under light anesthesia. Mice were monitored daily for signs of VSV infection, such as hind limb paralysis, decreased locomotion, and weight loss. Upon signs of end-stage illness, mice were euthanized and organs (brain, liver, spleen, lungs) were collected for histological analysis. (A) The percentage survival of mice infected with varying doses of wtO, rwt, or rM51R-M viruses. Two separate experiments were carried out using five mice per group. (B) Weight of mice infected with rwt and rM51R-M viruses. The weight of mice was measured 3× weekly following infection with 10^6 pfu of rwt and rM51R-M viruses. The experiment included five mice per group. (C) Virus titers in tissues of infected mice. Mice were intranasally inoculated with 10^6 pfu of rwt and rM51R-M viruses. At day 7 postinfection, animals were sacrificed, and organs (brain and lungs) were collected for determination of viral titers by plaque assay. Data represent the mean titer ± SD of 2–3 mice.

as high as 10^8 pfu, remained healthy for the course of the experiment (Fig. 9A). Statistical analysis indicated that the rM51R-M virus was significantly less pathogenic than both rwt ($P = 0.0053$) and wtO ($P < 0.0001$) viruses. Furthermore, these mice showed no signs of illness as indicated by a lack of weight loss (Fig. 9B) for at least 2 months, at which time they were sacrificed. In contrast, all mice infected with rwt virus showed signs of illness as indicated by statistically significant weight loss from day 6 postinfection as compared to mice infected with rM51R-M virus.

Viral titers in brain and lung collected from mice at day 7 postinfection were determined to analyze the presence of infectious virus in infected animals (Fig. 9C). Although we were unable to detect infectious virus in the lungs of VSV-infected mice at these times, brains of mice infected with each of the viruses had detectable levels of infectious virus. However, viral titers from the brains of rwt-infected mice were 4 logs lower than those obtained from brains of mice infected with wtO virus. Furthermore, brains of mice infected with rM51R-M virus had the lowest levels of infectious virus. These data indicate that although the rM51R-M virus was able to spread to the brain, it was less pathogenic than viruses containing wt M proteins.

Discussion

One of the underlying assumptions in the use of VSV as an oncolytic virus is that tumor cells have defective antiviral responses compared to the normal cells from which they were derived. Prostate tumors provide an excellent opportunity to test this hypothesis since prostate tumor cells, such as LNCaP and PC-3 cells, can be compared to benign prostate epithelial cells cultured from patient tissues. Results in this paper demonstrated that LNCaP cells were sensitive to infection and killing by VSV, while PC-3 cells were highly resistant in cell culture (Figs. 4 and 5). Furthermore, similar results were obtained upon VSV treatment of LNCaP and PC-3 tumor-bearing mice (Fig. 8). PC-3 cells were able to produce high levels of IFN (Fig. 2) and respond to the antiviral effects of IFN (Fig. 1), while LNCaP cells were not responsive to IFN. This difference may account in part for the resistance of PC-3 cells to VSV. Although this is an attractive hypothesis, our results indicate that the IFN-inducing rM51R-M virus grew to levels comparable to those of rwt virus in PC-3 cells (Fig. 3E). Therefore, the overexpression of IFN in rM51R-M virus-infected PC-3 cells does not activate an antiviral response to selectively attenuate rM51R-M virus infection. Based on these data, it is likely that antiviral factors other than or in addition to those activated by the IFN response pathway contribute to the resistance of PC-3 cells to VSV infection and killing. Despite the fact that LNCaP and PC-3 cells are both derived from metastatic prostate tumors, numerous molecular differences between these two cell types have been described

(Payaloor et al., 1999; Polek et al., 2003; Yang et al., 2003). Our future experiments will determine whether previously observed differences in these tumor cells are responsible for their differential susceptibility to VSV. Furthermore, additional candidate genes that are differentially regulated in these VSV-sensitive and -resistant cell lines will be identified by microarray comparisons.

It has generally been accepted that normal cells are more resistant to VSV-induced cytopathic effects than tumor cells (Obuchi et al., 2003; Stojdl et al., 2000). However, our data show that prostate epithelial cells are not inherently resistant to VSV infection, since both viruses produced infectious progeny and killed benign prostatic epithelial cells as well as LNCaP cells in single-cycle infection experiments (Figs. 4 and 5). These results are similar to those in mouse embryo fibroblasts that exhibit some susceptibility to VSV infection (Obuchi et al., 2003; Stojdl et al., 2003), but contrast with those in rat hepatocytes that appear to be nonpermissive for VSV growth (Ebert et al., 2003). The ability of VSV to infect benign prostate cells should not be a concern for the use of this virus for prostate tumor therapy, since normal prostatic tissue is surgically removed as part of the therapy. Furthermore, our results also show that the rM51R-M virus induced high levels of IFN production in benign prostate cells while the rwt virus did not (Fig. 5). In contrast to data obtained in single-cycle experiments, multiple-cycle infection experiments indicate that benign prostate cells are resistant to infection with rM51R-M virus (Figs. 6D and E), but not rwt virus, supporting the idea that the M protein mutant virus induces an effective antiviral response in infected benign prostate cells, thereby protecting surrounding uninfected cells from VSV-induced cell killing.

One of the principal cytopathic effects of VSV infection is the potent activation of apoptosis, which is the basis for its oncolytic activity. Recent studies from our laboratory have shown that at least two viral components are involved in the induction of apoptosis by VSV, M protein, and another viral product(s) that has not been identified (Kopecky et al., 2001). It was important to determine the relative importance of M protein for the induction of apoptosis in prostate tumor cells, because the role of M protein in the regulation of apoptosis during a virus infection is dependent on cell type. Studies with rwt and rM51R-M viruses have shown that in some cell types, such as HeLa cells, the wt M protein accelerates VSV-induced apoptosis (Kopecky et al., 2001). This is due to the ability of wt M protein to inhibit host gene expression, which rapidly induces apoptosis through activation of the mitochondrial pathway in these cells (Kopecky and Lyles, 2003). However, in other cell types, such as BHK cells, the rM51R-M virus induces apoptosis more rapidly than rwt virus, indicating that another viral product is the major inducer of apoptosis in these cells (Kopecky and Lyles, 2003; Kopecky et al., 2001). Experiments presented here show that there was no difference between rwt and rM51R-M viruses at inducing apoptosis in LNCaP and PC-3 prostate

tumor cells in single-cycle infection experiments (Figs. 4A and B). Similar results were seen in benign prostate cells infected with these viruses (Fig. 4C). These data indicate that the M protein plays little role in inducing apoptosis in VSV-infected prostate cells.

Laboratory strains of VSV have little if any pathogenicity for healthy adult humans (Richmond and McKinney, 1999). However, the potential for VSV to cause disease in cancer patients who may be immunocompromised has not been explored. Previous studies testing the effectiveness of VSV as an oncolytic virus have shown that immunocompromised mice are highly susceptible to lethal infection with wt VSV strains (Huneycutt et al., 1993; Stojdl et al., 2000). Since one of the main concerns regarding the use of viral vectors for tumor therapies is their safety in patients, our goal was to develop viruses based on VSV that were less pathogenic than their wt counterparts. Therefore, our idea was that IFN-inducing viruses, such as rM51R-M virus, would be safer in vivo due to their inability to suppress an effective antiviral response in normal tissue. Indeed, our data show that rM51R-M and rwt viruses were equally effective in reducing LNCaP tumor burdens in nude mice (Figs. 7 and 8), but rM51R-M virus had a dramatically reduced pathogenicity in both nude mice and immunocompetent mice (Figs. 7, 8, and 9). These results support our hypothesis that M protein mutant viruses would not only be effective oncolytic viruses for prostate tumor therapy due to their ability to kill tumor cells, but would also be safer viruses due to their ability to induce antiviral responses and spare normal cells. Similar strategies for enhancing the safety of VSV vectors include pretreating mice with IFN (Stojdl et al., 2000), engineering an IFN gene into the VSV genome (Obuchi et al., 2003), and using naturally occurring IFN-inducing mutants of VSV (Stojdl et al., 2003).

Several studies have demonstrated the effectiveness of VSV as an oncolytic agent for anti-tumor therapies in experimental animals with a variety of tumor types (Balachandran and Barber, 2000, 2001; Ebert et al., 2003; Huang et al., 2003; Obuchi et al., 2003; Stojdl et al., 2000, 2003). These tumors include p53-null C6 glioblastomas (Balachandran and Barber, 2001), myc and ras transformed cell lines (Balachandran and Barber, 2001), and human melanoma xenografts (Stojdl et al., 2000) in nude mice. In addition, VSV represses the growth of several different metastatic tumor models in immunocompetent mice (Huang et al., 2003; Obuchi et al., 2003; Stojdl et al., 2003) and hepatocellular carcinomas in rats (Ebert et al., 2003). Our results with the treatment of prostate tumor xenografts are consistent with these previous studies (Figs. 7 and 8). The effectiveness of VSV in reducing tumor volume varies considerably among these different tumor models. Our results showing that a single treatment of a well-established tumor derived from LNCaP cells with rM51R-M virus led to complete tumor regression in some cases without causing disease in nude mice stands among the most successful examples. One of the key issues in the treatment of prostate

tumors will be the ability to treat widespread metastatic tumors. In this regard, systemic treatment by intravenous inoculation with VSV has been shown to be as effective as intratumoral inoculation in different tumor systems (Balachandran and Barber, 2001; Stojdl et al., 2003), and we have confirmed this result with rM51R-M virus treatment of LNCaP cells (Figs. 7 and 8). This reflects the ability of VSV to spread systemically and seek out the tissues that are susceptible to virus infection (Reiss et al., 1998).

The fact that mice bearing tumors derived from PC-3 cells were resistant to treatment with VSV indicates that VSV is not effective at treating all prostate tumors (Fig. 8). Thus, any future strategy for using VSV for prostate tumor therapy is likely to involve testing individual tumors for their susceptibility to virus infection. We are in the process of analyzing patient tumors for their susceptibility to VSV infection and killing. Our preliminary results show that primary cultures from tumor regions of human prostatectomy specimens exhibit a phenotype intermediate between that of LNCaP and PC-3 cells in their susceptibility to VSV infection and apoptosis. Therefore, it is possible that prostate tumors isolated from patients exhibit a range of sensitivity to VSV infection and killing. Future studies will allow us to determine the degree of differences present in prostate tumors and develop viruses that will more successfully treat such tumors.

Previous experiments to determine the efficacy of VSV as a vector for anti-tumor therapies used naturally occurring wt and mutant strains of VSV in mice (Balachandran and Barber, 2000, 2001; Stojdl et al., 2000, 2003). In this study, we tested the effectiveness of less pathogenic recombinant strains of VSV as agents for prostate tumor therapy. The recombinant background resulted in a significantly less pathogenic virus than the laboratory adapted wtO virus (Fig. 9A) and therefore offers great promise as a backbone from which additional viral vectors will be constructed. Furthermore, although LNCaP tumor volume in rwt virus-treated mice decreased over time similar to results obtained in mice treated with rM51R-M virus (Figs. 7 and 8), it is possible that in certain cases rM51R-M virus may be prematurely cleared by the host before it can effectively spread to distal tumor sites. Therefore, our future studies will include incorporating M protein mutations in the virus that would confer varying degrees of pathogenicity in animals. These M protein mutant viruses would further be analyzed as potential vectors for therapies of tumor that may be more resistant to rM51R-M virus. One such M protein mutation is the N163D substitution that is conserved in a heterogeneous viral population from persistently infected cells (Ahmed and Lyles, 1997). The N163D M protein exhibits an intermediate phenotype between wtO M protein M51R-M in its ability to inhibit host gene expression (Ahmed and Lyles, 1997). By testing viruses with such mutations, this will allow us to determine the balance necessary between viral and host factors for the development of recombinant viruses for effective tumor therapies.

Materials and methods

Cells and viruses

LNCaP and PC-3 tumor cells were from the American Type Culture Collection and were grown in RPMI 1640 containing 10% FBS. Primary human prostatic epithelial cells were isolated from prostatectomy specimens at Wake Forest University School of Medicine by modification (Barreto et al., 2000; Rao et al., 2002) of the method of Peehl (1992) and Peehl et al. (1988). Briefly, a small piece of tissue from each specimen was removed using a sterile cork borer. The prostatectomy specimen was inked with a color-coded scheme to trace the pathological origin of the culture specimen. The tissue plug was minced with scissors and a sterile razor blade and digested overnight with 40–60 U collagenase/ml of medium PFMR-4A plus growth factors and hormones. To remove the majority of the stromal cells, the tissue was rinsed twice with 5 ml HEPES-buffered saline (HBS) and centrifuged. The digested tissue was inoculated into a 60-mm tissue culture dish coated with collagen type I (Vitrogen 100, Cohesion, Palo Alto, CA) and grown in medium PFMR-4A supplemented with growth factors and hormones. The cells that grew out from the tissue were aliquoted and stored in liquid nitrogen. Adjacent tissue blocks were used to determine the pathological origin of the culture. Only samples greater than 95% of a particular histological type (i.e., benign or cancer) were used. Heterogeneous specimens were not used. The frozen aliquots were thawed to produce secondary cultures, which were grown in medium MCDB 105 supplemented with growth factors and hormones (Peehl, 1992; Peehl et al., 1988). Prostatic epithelial cells derived by these methods are designated as normal prostatic epithelial cells due to their inability to form tumors or benign structures in xenograft models, and due to the lack of cytogenetic abnormalities that are found in cells derived from cancer areas of the prostate. In addition, these cells were tested for 1alpha hydroxylase, whose expression is reduced in tumor cells.

Nomenclature for prostatic epithelial cell strains is "WFU" followed by the strain number (in serial order of acquisition) and then the histology of origin (PZ, peripheral zone; BPH, benign prostatic hyperplasia).

Wild-type VSV (Indiana serotype, Orsay strain) was grown in BHK cells as described previously (Lyles and McKenzie, 1997). The recombinant viruses, rwt and rM51R-M, were isolated from infectious VSV cDNA clones and virus stocks were prepared in BHK cells as described (Kopecky et al., 2001).

Responsiveness to IFN

LNCaP, PC-3, and HeLa cells were plated onto 96-well dishes and pretreated with different concentrations of IFN (6.4–20000 IU/ml). Cells were incubated overnight and challenged with wtO virus at an MOI of 0.015 pfu/cell for LNCaP and HeLa cells, and an MOI of 30 pfu/cell for PC-3 cells. It was important to use different MOIs for PC3 cells in order to obtain cytopathic effects within the time scale of the assay. Furthermore, the use of 96-well dishes rather than 6-well dishes was important for optimizing this assay. At varying times postinfection, live cells were measured by an MTT assay (Cell Proliferation Kit 1; Roche Diagnostics, Indianapolis, IN) for cell viability. Controls included IFN-untreated cells infected with VSV and IFN-treated cells that were not challenged with VSV. Data are the averages of two separate experiments.

Interferon bioassay

To determine the IFN activity produced by cells infected with wt and mutant viruses, supernatants (100 μl) were collected from LNCaP, PC-3, and benign prostate cells infected with wt and mutant viruses for 6, 12, 24, 30, and 36 h. Infectious virus was inactivated by acid treatment, the acid was neutralized, and serial dilutions were incubated with HeLa cells in 96-well plates overnight at 37 °C. To construct a standard curve dose response, cells were incubated with serial 5-fold dilutions of IFN (Universal type I IFN, PBL Biomedical Laboratories, New Brunswick, NJ). The samples were aspirated and cells were challenged with wt VSV at 2.24 × 10^4 PFU/ml in 100 μl of media. Controls included cells infected with VSV alone and cells that were not challenged with VSV. Cells were incubated overnight at 37 °C, media were aspirated, and cells were fixed with 95% ethanol. Cells were then stained with a 0.1% crystal violet solution in methanol. Absorbance was read at 550 nm on an ELISA reader. IFN levels greater than 150 IU/ml were detectable by this bioassay. Data are the averages of two independent experiments.

G protein surface expression

The efficiency of G protein cell surface expression during VSV infection in LNCaP, PC-3, and benign prostate cells was determined by flow cytometry analysis of infected cells. Cells were seeded to approximately 70% confluence in 35-mm dishes and infected with wt and mutant viruses at a multiplicity of 10 pfu/ml for 4 and 8 h in RPMI containing 2% FBS for LNCaP and PC-3 cells, and MCDB complete for benign prostate cells. Following infection, cells were washed twice and then incubated for 1 h with 0.1 M glycine–10% BSA in PBS at 4 °C. Surface-expressed G protein was labeled with anti-G protein antibody I1 (Lefrancois and Lyles, 1982) at a 1:500 dilution for 1 h. Cells were washed three times and incubated with a goat anti-mouse immunoglobulin G secondary antibody conjugated to fluorescein (ICN Biomedicals, Inc.) at a dilution of 1:200 for 1 h at 4 °C. Cells were washed three times, fixed for 10 min in 4% formaldehyde at 4 °C, and collected in 1 ml of 10% BSA. G protein cell surface fluorescence was

quantitated with a Becton Dickinson FACSCaliber flow cytometer.

Growth curves

LNCaP, PC-3, and benign prostatic cells in 35-mm dishes were infected with viruses containing rwt or rM51R-M viruses at a multiplicity of 10 pfu/cell (single step growth curve) or 0.1 pfu/cell (multiple step growth curve) in RPMI 1640 containing 2% FBS (LNCaP and PC-3 cells) or MCDB 105 complete (benign prostatic cells). At 1 h postinfection, the media were aspirated, cells were washed twice with PBS, and fed with 2 ml of the appropriate media. At the indicated times postinfection, 100 μl of media was removed from the dishes and stored at −70 °C. The yield of virus was determined by plaque assays on BHK cells and was expressed as pfu/ml.

Apoptosis assay

The percentage of infected cells undergoing DNA fragmentation characteristic of apoptosis was determined by TUNEL analysis (In Situ Cell Death Detection Kit; Roche Diagnostics). LNCaP, PC-3, and benign prostate cells were infected with rwt and rM51R-M viruses at a multiplicity of 10 pfu/cell. At 24 and 48 h postinfection, cells were harvested, fixed in paraformaldehyde, and permeabilized. Cells were incubated with the TUNEL reaction mixture containing TdT and fluorescein-dUTP to free 3′ OH ends in the DNA. The incorporated fluorescein was analyzed and quantitated by flow cytometry on a Becton Dickinson FACSCalibur flow cytometer.

Cell viability assay

LNCaP, PC-3, or benign prostate cells were infected with wt and mutant viruses at an MOI of 0.1 or 10 pfu/cell. At different times postinfection, live cells were measured by an MTT assay (Cell Proliferation Kit 1; Roche Diagnostics) according to the manufacturer's instructions.

Tumor treatment

LNCaP and PC-3 cells were harvested from semiconfluent cultures and cell viability was determined using trypan blue exclusion. Cells with greater than 90% viability were used for the experiments. Cells were suspended at 2×10^6 cells/0.2 ml of ice-cold Matrigel obtained from Collaborative Biomedical Products (LNCaP), or in 0.2 ml of RPMI (PC-3 cells), and were injected subcutaneously in the flank of BALB/c nude mice. Animals were monitored for tumor development three times a week by palpation of the injection site. Animals with palpable tumors had their tumor volume measured by calipers and the volume calculated using the formula Volume = (width)^2 × length/2. At 3–4 weeks

after tumor cell injection, the tumor-bearing animals were randomly separated into three experimental groups. Animals were injected with VSV either intratumorally (1×10^8 pfu) or intravenously (2×10^8 pfu) in culture medium. Culture medium alone was used as negative control. Tumor volume was measured daily with calipers, as described above, and animal mass was also measured. If the animals showed irreversible symptoms of VSV infection (usually around 6 days postinfection) or signs of end-stage illness as indicated by IACUC guidelines of Wake Forest University Health Sciences, they were sacrificed, and the tumor and selected tissues (brain, lungs, spleen, and liver) were harvested for histological analysis.

LD$_{50}$ determination

Intranasal inoculation of 6-week-old BALB/c male mice (Charles River Laboratories) with varying titers of wt and mutant viruses was performed under anesthesia using avertin (2,2,2 tribromoethanol and *tert*-amyl-OH). Two separate experiments were conducted using five mice per virus dose. For the first group, mice were inoculated with a total volume of 20 μl containing 10^2–10^6 pfu of wtO, rwt, and rM51R-M viruses. For the second group, mice were infected with 10^5–10^7 pfu of rwt and 10^6–10^8 pfu of rM51R-M viruses. Mice were monitored daily for signs of VSV infection, such as hind limb paralysis. Upon signs of end-stage illness, mice were sacrificed and tissue (brain, lung, spleen, and liver) was harvested for histological analysis.

Immunohistochemistry

Harvested tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin for histological examination or used in immunohistochemical staining. For immunohistochemical staining, cells were fixed in descending series of ethanol washes, quenched with 0.3% peroxide in PBS, and blocked in 5% goat serum. Serial sections were incubated overnight with antibodies against the viral envelope glycoprotein (rabbit anti-G, Research Diagnostics, Inc., Flanders, NJ). Secondary antibody (biotinylated anti-rabbit from BioGenex Supersensitive kit) was incubated on sections at room temperature for 30 min. Primary antibody detection was accomplished using a streptavidin alkaline phosphatase detection kit (Supersensitive Detection Kit, Biogenex, San Ramon, CA). Vector Red Substrate kit No.1 for alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA) was used to visualize the antibody–antigen complex. Nuclei were counterstained using Mayers hematoxylin. Negative controls consisted of histologic sections processed without the addition of primary antibody, but incubated instead with 1% goat serum or mouse IgG (Reagent Grade, 0.33 mg/ml, Sigma, Saint Louis, MO).

Viral titers

Tissues from VSV-infected mice were harvested and homogenized in media containing penicillin and streptomycin. The homogenate (100 μl) was used to determine the yield of virus by plaque assays on BHK cells. Data are the average viral titers in tissue from two animals and are expressed as pfu/tissue.

Statistical analysis

Chi-square tests of heterogeneity were used to test whether there were significant differences in rate of death after 14 days of exposure between wt and mutant viruses. To account for the increased risk with increased dosage levels and the fact that there were no observed deaths for rM51R-M virus, a permutation-based test was used to evaluate the statistical significance of the differences in the observed rates of death. Specifically, for each pairing of viruses, a 2 × 2 contingency table was constructed and a chi-square test statistic was calculated from the observed data. Random replicate data sets were generated by randomly permuting deaths between viruses. To address the impact of different dosages, we fixed the marginal counts of deaths and the total number of observations for each virus at each dosage level and randomly permuted deaths between two viruses at each dosage level. Finally, a chi-square statistic was constructed for each random replicate data set and a *P* value was calculated as the proportion of random replicates that had an equal or greater chi-square test statistic than the observed test statistic.

We used mixed linear models (PROC MIXED; SAS windows version 8, SAS Institute, Cary, N.C.) to assess differences in tumor size overtime upon intratumoral treatments. Data were used from three separate experiments, which were treated as random effect in the models. Specifically, we tested whether there were significant differences in percent change, from baseline, of tumor size between rwt and rM51R-M viruses after 7 and 14 days, adjusting for experiment. We also tested whether changes in tumor size were significant between 14 and 7 days for each virus. Least square means were calculated for each virus after 7 and 14 days.

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